The interplay of oxidative stress and inflammation in atherosclerosis: an epidemiologic approach

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The interplay of oxidative stress and inflammation in atherosclerosis: an epidemiologic approach

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door

Leonard Peter van der Zwan

geboren te Ten Boer

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copromotoren:	dr. T. Teerlink
	dr. P.G. Scheffer

Aan mijn ouders

Contents

Chapter 1

Introduction and outline of the thesis

Prevalence of cardiovascular disease and diabetes mellitus

Cardiovascular disease (CVD) is the second most prevalent disease in men and the third most prevalent in women.[1] Moreover, it is the primary cause of death in both men and women.[2] On an annual basis an estimated 17.1 million persons die because of CVD, which constitutes 29% of all mortality.[1]

In subjects with diabetes mellitus the risk for developing CVD morbidity is about 2.5 times increased.[3] In subjects with diabetes, the risk of macro- and microvascular complications is increased, endothelial function is impaired, and arteries are stiffened.[4-6] Furthermore, diabetes mellitus has been linked to key features of atherosclerosis development: dyslipidemia, inflammation, and oxidative stress.[7-11] Type 1 diabetes is caused by low or no insulin production, whereas type 2 diabetes (T2DM) is characterized by insensitivity of tissues to insulin, leading to a reduced uptake of glucose from the bloodstream. T2DM is the most prevalent form of diabetes mellitus (>90%) and its prevalence increases with age. Currently, diabetes affects more than 220 million people worldwide. Annually approximately 1.1 million people die due to complications of diabetes, which corresponds to a 2-fold increased risk for CVD mortality.[12]

Hypotheses in atherosclerosis disease development

Atherosclerosis is a progressive disease that encompasses several processes (Figure 1). The arterial layout from lumen to the outside of an artery comprises of endothelium, intima, media, and adventitia. The endothelial layer forms a barrier for components of the blood stream like blood cells and relatively large lipoproteins like chylomicrons and very low-density lipoproteins (VLDL). Low-density lipoprotein (LDL) and high-density lipoprotein (HDL) particles may pass this barrier freely, while inflammatory cells can pass this barrier only actively (Figure 2).[13,14] In healthy vessels the intima is a thin layer.[15] The media consists of smooth muscle cells, and the adventitia forms the cover of the artery.[15]

In 1858, Virchow, who is generally considered the founder of cellular pathology, gave a lecture on atheroma.[16] In this lecture he described increased numbers of cells, fat granules in cells and “lipoid substances” in atheromatous thickening of the vessel wall. He concluded that blood components had entered the intima and two processes could be distinguished: fatty degeneration leading to

perishing of the vessel wall and a series of inflammation-like changes. Knowledge about atherosclerosis was further extended by Anichkov (Anitschkow). He worked as a physician in the Russian army during the First World War and worked as a scientist/physician in both Russia and Germany.[17] In 1913, Anichkov studied the role of dietary cholesterol in atherosclerosis by feeding rabbits cholesterol.[18] He observed piling up of cholesterol in the intima of the aorta as a result of the cholesterol-rich diet after 6-8 weeks.[18] At the time Anichkov presented his observations he had no knowledge about lipoproteins.[19] In fact, lipoproteins were first noticed a couple of years after his discovery and it would take decades of improvements in analytical techniques, such as the introduction of ultracentrifugation, before two major lipoprotein classes were characterized in 1949.[19] Moreover, it was not until 1950 that Gofman and coworkers

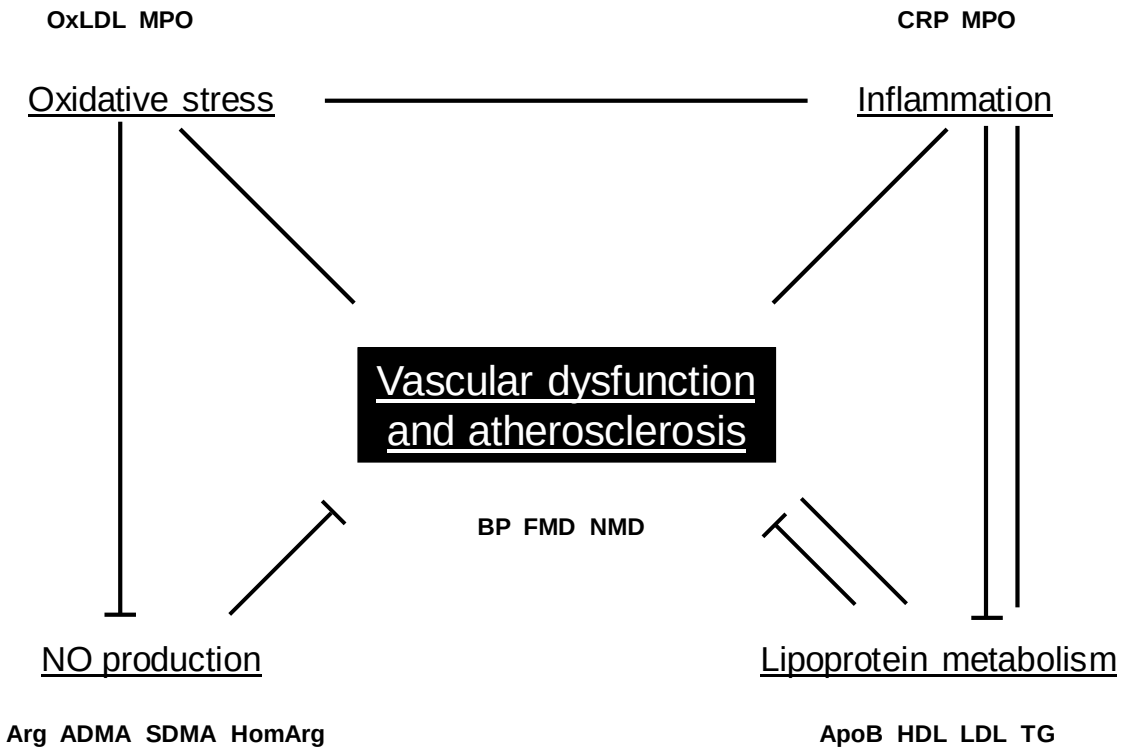

Figure 1. A schematic graph showing processes involved in vascular dysfunction and atherosclerosis.

Measured markers reflecting the specific processes are in bold font. Arrows represent stimuli, blunt-end lines inhibitions. Processes are underlined. OxLDL, oxidized low-density lipoprotein; MPO, myeloperoxidase; CRP, C-reactive protein; Arg, arginine; ADMA, asymmetric dimethylarginine; SDMA, symmetric dimethylarginine; HomArg, homoarginine; ApoB, apolipoprotein B-100; HDL, high-density lipoprotein; LDL, low-density lipoprotein; TG, triglycerides; BP, blood pressure; FMD, flow-mediated dilation; NMD, nitroglycerine-mediated dilation.

came up with the idea that lipoproteins might be involved in atherosclerosis.[19] Gradually improved analytical ultracentrifugation techniques –such as described by Havel, Eder, and Bragdon in 1956– would lead to better insight into the role of lipoproteins in cholesterol transport and atherosclerosis.[19,20]

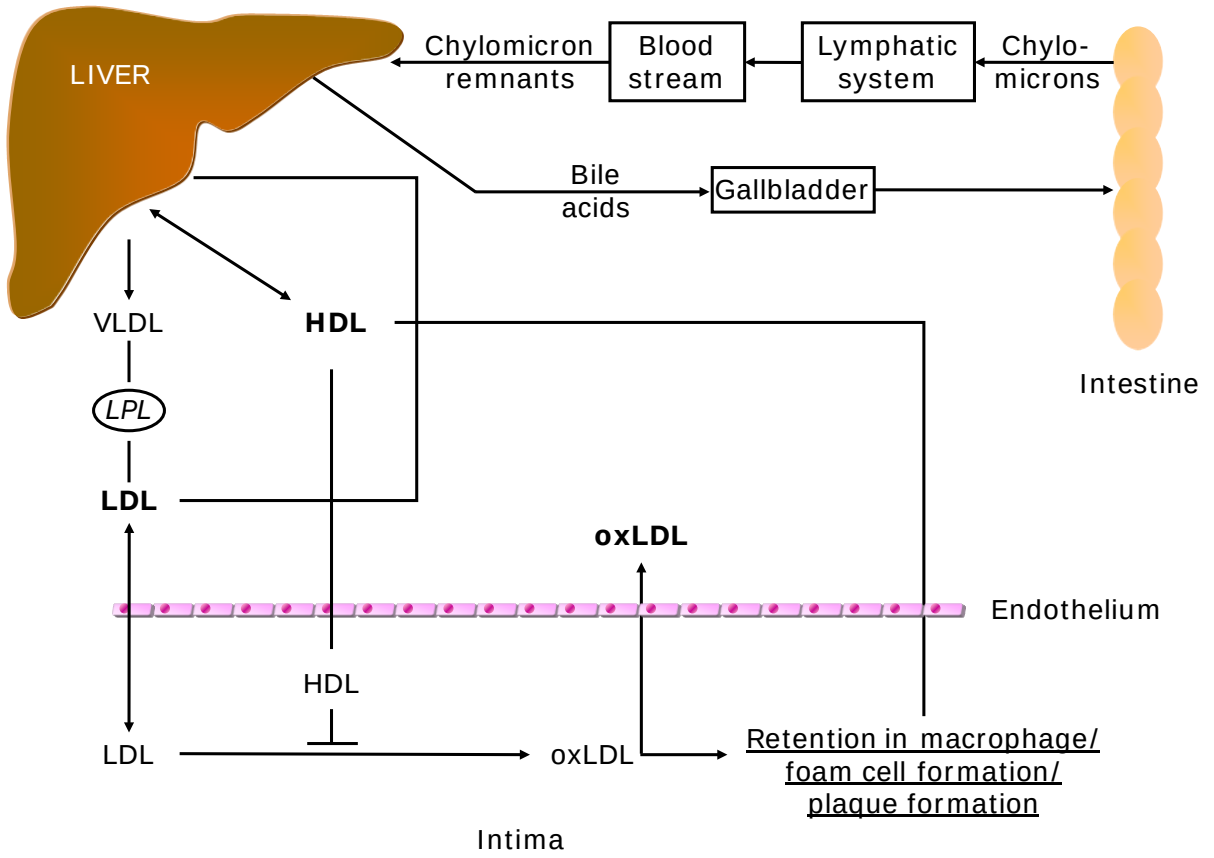

Figure 2. **Simplified schematic overview of lipoprotein metabolism.**

Dietary lipids are absorbed by the intestine, where they are assembled into triglyceride-rich chylomicrons that are transported via the lymphatic system to the blood. In the circulation the triglycerides from the chylomicrons are rapidly degraded by lipoprotein lipase (LPL) to supply peripheral tissues with fatty acids. The remaining chylomicron remnants are cleared by the liver. The liver produces and secretes very-low-density lipoproteins (VLDL) into the blood stream. By subsequent LPL-catalyzed hydrolysis of triglycerides VLDL particles become smaller and are ultimately transformed into low-density lipoprotein (LDL). In the circulation LDL is the main carrier of cholesterol which it distributes to peripheral tissues. LDL can pass the endothelial layer of the artery wall where it may be modified and oxidized. Once LDL is highly oxidized (oxLDL) it is taken up by macrophages, which, if taking up oxLDL in excess, turn into foam cells. Piling up of foam cells leads to formation of fatty streaks, which subsequently may progress into atheroma. The liver also produces high-density lipoprotein (HDL). By taking up cholesterol throughout the body, nascent HDL particles grow and change shape into spherical HDL particles. HDL particles may then return to the liver (reverse-cholesterol transport). HDL particles have other functions as well: protection of LDL from oxidation, suppression of inflammation, and inhibition of foam cell formation. Bile acids are synthesized in the liver from cholesterol and secreted into the gallbladder, from where they pass into the intestine. Measured compounds are in bold. Processes are underlined.

In 1973, the “response to injury hypothesis” was introduced by Ross and Glomset.[21,22] They hypothesized that injury to the endothelium causes increased inflow of LDL particles in the vessel wall, followed by leukocyte attraction, foam cell formation, platelet adherence, and migration of smooth muscle cells into the intima.[22] In 1995, the alternative “response-to-retention hypothesis” argued that retention of lipoproteins in the vessel wall caused by interaction with proteoglycans of the subendothelium is the key event in atherosclerosis initiation.[23] However, only the oxidative modification theory explained the excessive LDL uptake by macrophages.[24-27] This latter theory hypothesizes that LDL is modified in the microenvironment of the intima. Subsequently, the modified LDL initiates chemotaxis of monocytes from the blood stream into the vessel wall. Reactive compounds and pro-oxidative enzymes released by activated leukocytes may then cause extensive oxidation of LDL. Uptake of the highly oxidized LDL particles by macrophages then leads to foam cell formation. The piling up of these foam cells causes the formation of fatty streaks that may subsequently evolve into atherosclerotic plaques.[15]

Recently, other factors involved in atherosclerosis such as the anti-atherogenic functions of HDL (Figure 2), inflammatory processes (Figure 3), and nitric oxide (NO) signaling (Figure 4) have (re)gained interest.

HDL transports cholesterol to the liver where cholesterol is converted to bile acids and excreted into the intestine. In addition, HDL distributes enzymes that break down pro-inflammatory compounds, inhibits migration of inflammatory cells into the vessel wall, and serves as a sink for oxidants and oxidant-producing enzymes that may damage LDL particles and vascular tissues (Figures 2 and 3).[28-31] However, HDL loses its protective functions when modified by highly reactive compounds.[30,32,33]

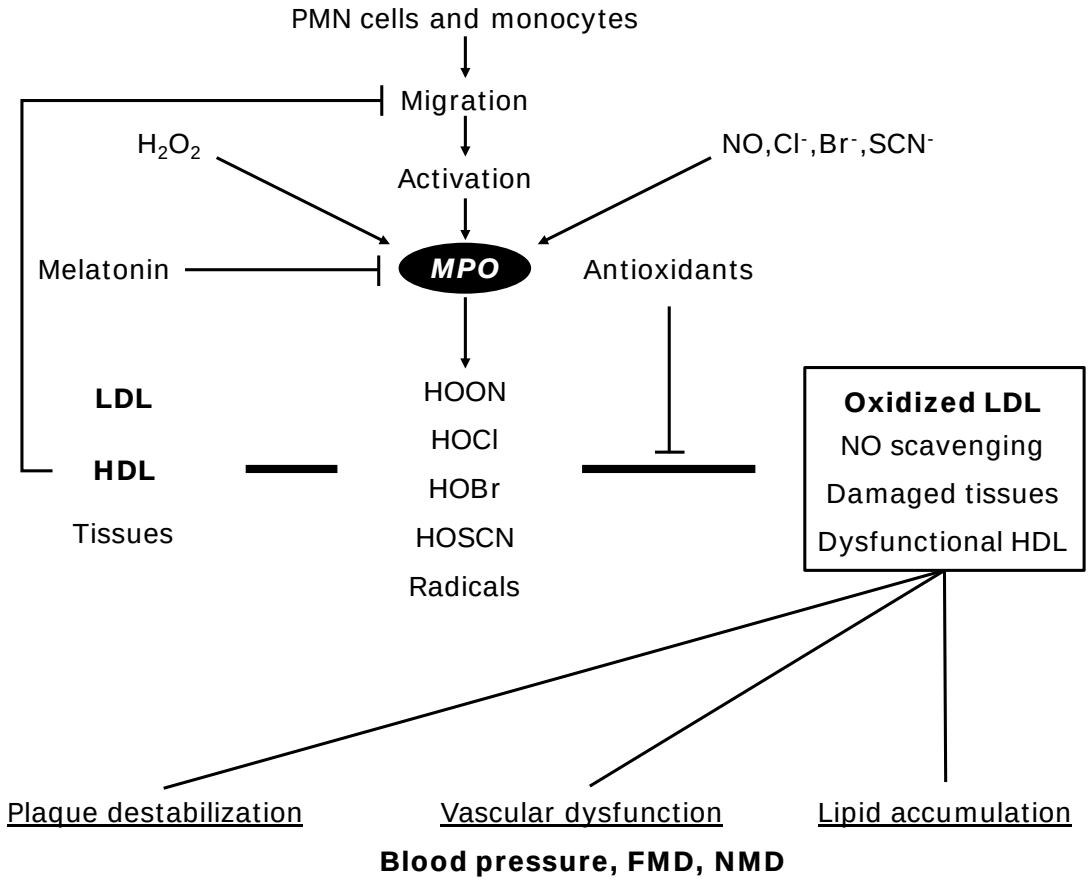

Figure 3. **The role of myeloperoxidase in atherosclerosis.**
Polymorphonuclear (PMN) cells and monocytes may migrate to or into the arterial wall. Here, upon activation, monocytes differentiate into macrophages and both PMN cells and monocytes/ macrophages may release the enzyme myeloperoxidase (MPO). MPO then uses hydrogen peroxide as cosubstrate and (pseudo) halogen ions (NO, Cl^-, Br^-, SCN^-) as substrate to form MPO-derived reactive species (MDRS). These MDRS may then react with lipoproteins or components of the vascular matrix. LDL thus turns into oxidized LDL, HDL becomes dysfunctional, and vascular tissues get damaged, resulting in vascular dysfunction, lipid accumulation, plaque formation, and ultimately plaque destabilization. The adverse effects of MPO may be counteracted by antioxidants that reduce the amount of hydrogen peroxide or scavenge MDRS and also by direct inhibition of MPO by melatonin. LDL, low-density lipoprotein; HDL, high-density lipoprotein; FMD, flow-mediated dilation; NMD, nitroglycerine-mediated dilation. Measured biomarkers are in bold. Processes are underlined.

NO is a gaseous molecule involved in cell signaling and NO produced by the endothelium may rapidly diffuse to nearby vascular smooth muscle cells. There it stimulates activity of the enzyme soluble guanylate cyclase and the resulting increased concentration of the second messenger cGMP induces vasodilation by relaxation of the smooth muscle cells (Figure 4).[34] This powerful vasodilatory action together with its short half life makes NO an important regulator of vascular tone, organ perfusion, and blood pressure.

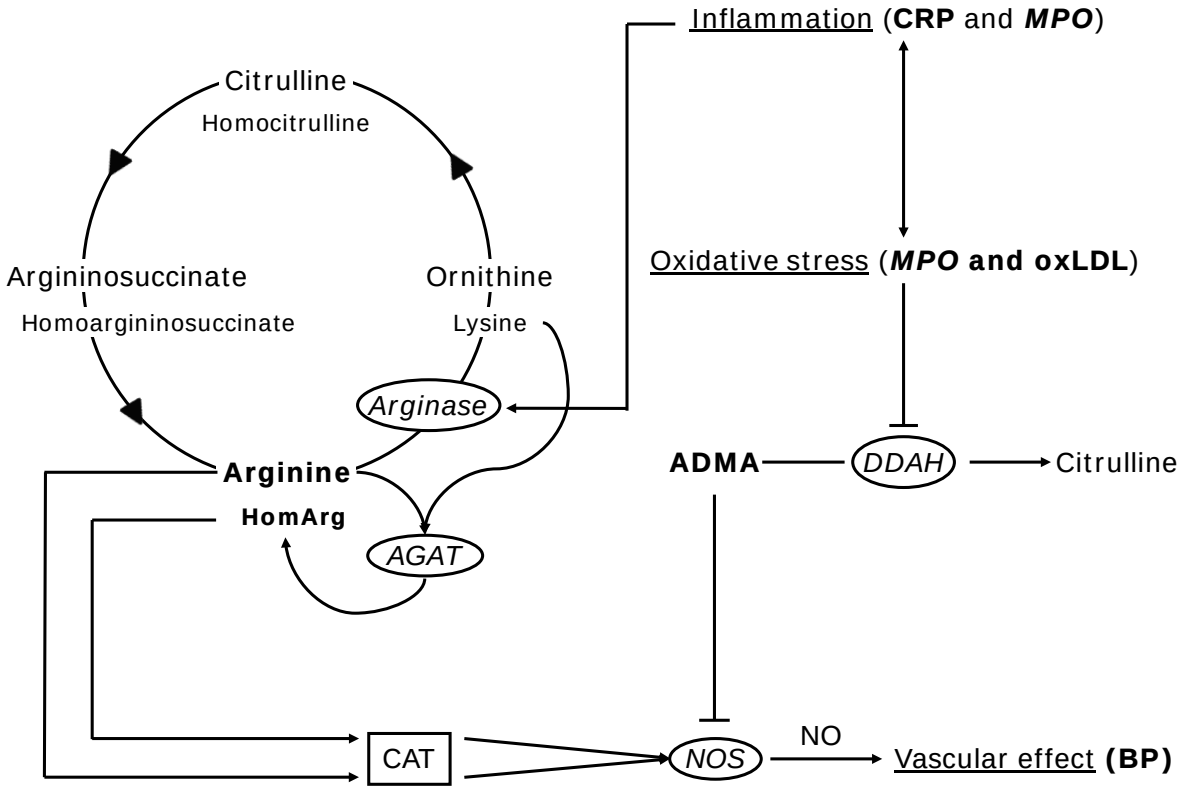

Figure 4. **Role of arginine, homoarginine, and asymmetric dimethylarginine in nitric oxide signaling and vascular (dys)function.**
Arginine is synthesized in the the urea cycle. Homoarginine may be synthesized in an alternative urea cycle in which arginine, ornithine, citrulline and argininosuccinate are replaced by homoarginine (HomArg), lysine, homocitrulline, and homoargininosuccinate, respectively. Another synthesis route of HomArg is a transamidination reaction catalyzed by L-arginine:glycine transamidinase (AGAT) using arginine as donor of an amidino-group and lysine as acceptor. Arginine and HomArg can be competitively transported into the cell by cationic amino acid transporters (CAT). In the cell arginine and HomArg are competing substrates of nitric oxide synthase (NOS). Nitric oxide (NO) is a signaling molecule for the smooth muscle cells of the artery to relax and thus dilate the vessel. Asymmetric dimethylarginine (ADMA) is a competitive inhibitor of NOS. ADMA is actively degraded by dimethylarginine dimethylaminohydrolase (DDAH), but this enzyme is inactivated under conditions of oxidative stress. Inflammation, which is associated with oxidative stress as reflected by high levels of MPO and oxidized LDL (oxLDL), may thus lead to reduced NO production by increasing levels of ADMA. In addition, inflammation may lead to upregulation of arginase, leading to increased consumption and reduced levels of arginine, thereby further reducing NO output. Measured biomarkers are in bold and enzymes are in italic. Processes are underlined.

Next to vasodilation, endothelial NO also inhibits adhesion of circulatory cells to the endothelium and proliferation of vascular smooth muscle cells. Finally, NO also scavenges oxidants and together these actions of NO have an inhibitory effect on atherosclerosis development.[35] Although NO is generally anti-atherogenic, reaction of superoxide with NO results in formation of the highly damaging peroxynitrite radical and NO can also be used as substrate by myeloperoxidase (MPO) to make highly-reactive pro-atherogenic compounds.[33,36,37]

Cardiovascular risk markers

Over the past decades, knowledge about the etiology of atherosclerotic disease has greatly improved, but we still do not fully understand the underlying mechanisms and their interplay. Investigation of biomarkers reflecting the various processes involved in atherosclerosis may further our insight into these processes and their interactions.

A biomarker is defined as: “a characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention”.[38] Biomarkers can be divided into antecedent, screening, diagnostic, staging, and prognostic markers.[38-40] Antecedent markers predict the risk of developing a disease, while screening biomarkers may detect subclinical disease. Diagnostic biomarkers may identify overt disease. Staging biomarkers determine the severity of disease and can thus be used for monitoring patients. Finally, prognostic biomarkers are antecedent biomarkers that are modifiable by disease treatment. It should be noted that the term biomarker is not equivalent to risk factor. A biomarker is only a risk factor if it is associated with disease because it is in the causal pathway leading to disease. A biomarker that is associated with disease without being causally linked is often called a risk marker.[38]

In clinical studies, biomarkers have been used as surrogate endpoints instead of hard endpoints like mortality. Vascular dysfunction may be considered a surrogate endpoint, because of epidemiologic and pathophysiologic proof.[38,41,42] Blood pressure is a hard endpoint, but also a prognostic biomarker for CVD-related mortality.[43] Sometimes the estimation of disease risk can be improved by taking into account the information of several biomarkers. For example, the Framingham risk score uses age, LDL-cholesterol, HDL-cholesterol, blood pressure, smoking habits, and diabetes to calculate the risk and explains a large portion of cardiovascular events in the general population.[43] Notably, traditional risk factors are insufficient in explaining the increased risk for CVD-related mortality in type 2 diabetes.[44,45] Therefore, there is still a need for additional biomarkers. But even the study of biomarkers that do not improve disease risk estimation is of value, because it may yield novel insights in pathophysiological processes.

An example of a thoroughly evaluated novel (non-classical or non-traditional) CVD biomarker is C-reactive protein (CRP). CRP is an acute phase protein that is

mainly of hepatic origin and released into the circulation during a broad range of inflammatory processes.[46,47] Therefore, CRP is not specific for CVD risk estimation. CRP has also been evaluated in patients with diabetes mellitus and was found to be elevated.[48] Whether CRP is causally linked to CVD development is still subject of debate. CRP may be one of the signaling molecules initiating low-grade inflammation of the vessel wall, which starts or accelerates the atherosclerotic process. In support of this theory, CRP levels have been found to be positively associated with CVD in several studies and CRP has been found in atheroma.[49-51] However, CRP is predominantly produced by the liver and the liver is also involved in lipid metabolism. Therefore, the observed positive association between CRP and CVD may be due to confounding, i.e. the result of a causal relation between changes in lipid metabolism and CVD risk. Moreover, the results of recent Mendelian randomization studies do not support a causal role of CRP in CVD development.[52,53] Nonetheless, CRP is a useful biomarker for inflammation and may as such be of use in investigating the involvement of general inflammation in CVD.

Biomarkers investigated in the present thesis

Inflammation, oxidative stress, lipoprotein profiles, and NO homeostasis are thought to play a role in atherosclerosis (Figure 1). Therefore, we evaluated biomarkers of inflammation (CRP and myeloperoxidase [MPO]), oxidative stress (oxidized LDL [oxLDL] and MPO), lipid profiling (HDL-cholesterol [HDL-c], LDL-cholesterol [LDL-c], apolipoprotein B-100 [ApoB], and triglycerides [TG]), and NO homeostasis (arginine, methylated arginines, and homoarginine). Here, these markers will be discussed briefly.

Oxidized LDL

Because LDL is a complex particle consisting of several thousand molecules, oxidative stress may lead to myriads of different oxidative modifications. Therefore, oxidized LDL is an ambiguous term that does not refer to a single well-defined entity. In the literature, the term oxidized LDL has been used for: in vitro tests to measure the susceptibility of LDL to oxidation, tests determining oxidized phospholipids on LDL particles, and tests determining the oxidized protein moiety of LDL.[54-57] Measurement of the resistance of LDL against oxidation is generally performed by

exposure of isolated LDL to transition metal ions. The metal ion will catalyze oxidation leading to formation of conjugated dienes, which can be monitored by UV absorption detection.[58,59] Oxidants that oxidize lipids may sometimes, but not always, be oxidators of amino acids and vice versa. Therefore, different assays may lead to different outcomes, which do not necessarily need to correlate. For the studies described in this thesis we used an ELISA based on the monoclonal antibody 4E6, which recognizes apolipoprotein B-100 with at least 60 lysine residues modified by aldehydes. This antibody has indicated the presence of oxidized LDL in pig atheroma, and with this assay increased oxLDL concentrations have been found in humans with acute myocardial infarction.[60,61]

LDL oxidation is thought to occur in the vessel wall (Figure 2). To explain its presence in plasma it has been suggested that some oxLDL might escape scavenging by macrophages and return to the blood stream. An alternative explanation is that oxLDL leaks from atherosclerotic plaques into the blood stream. This latter mechanism would also explain the observed high plasma levels of oxLDL in subjects with acute myocardial infarction compared to stable myocardial infarction.[61]

Myeloperoxidase

Myeloperoxidase (MPO) is a 150 kDa glycated protein. In the presence of hydrogen peroxide it converts halogens (Cl^-; Br^-) or pseudo halogens (SCN^-; NO) into highly reactive substances (HOCl; HOBr; HOSCN; NOO^-).[62-65] These highly reactive compounds kill microorganisms and as such MPO helps to protect the human body against infectious diseases.[66] MPO is found in monocytes/macrophages and polymorphonuclear (PMN) cells. In atherosclerosis these cells are attracted to the inflamed part of the artery, where monocytes subsequently enter the intima, differentiate, and release MPO (Figure 3). At the luminal side of the endothelium PMN cells also release MPO, which subsequently enters the intima through transcytosis.[67] MPO release can be triggered by a variety of mechanisms. In human atheroma, granulocyte macrophage colony-stimulating factor selectively regulates MPO release by macrophages.[68] CRP is also capable of triggering release of MPO from polymorphonuclear cells and monocytes.[69] Additionally, it has been shown that

heparin induces MPO release from cells and liberates MPO from the endothelium.[67,70-72]

The MPO-derived reactive substances may oxidize LDL, thereby turning it into a ligand of scavenger receptors on macrophages. If uptake of oxidized LDL is excessive these macrophages may evolve into foam cells. OxLDL also is a signal to enhance inflammation and thus oxidation of LDL by MPO may result in boosted inflammation. When HDL is oxidized by MPO it loses its anti-oxidative and anti-inflammatory properties. Moreover, interaction of HDL particles with receptors on macrophages becomes impaired, resulting in a reduced reverse cholesterol transport (Figures 2 and 3).[73-77] Reactive products of MPO may also destabilize plaques, which may result in plaque rupture.[68,78] MPO may thus be involved in the initial phase of atherosclerosis as well as in turning late-stage atherosclerosis into acute cardiovascular events.

Arginine, homoarginine and methylated arginines

Arginine is a semi-essential amino acid. The human body can synthesize arginine, but under conditions of increased demand additional arginine from proteolysis of dietary proteins is required. Besides arginine, homoarginine is also found in human blood, albeit at much lower concentrations. The in vivo synthesis and degradation of homoarginine may result from transamidination reactions (Figure 4).[79,80] L-arginine:glycine amidinotransferase (AGAT), a key enzyme in the creatine synthesis route, may synthesize homoarginine by transferring an amidino group from arginine to lysine (Figure 4). Another possible route of homoarginine formation may be by synthesis from lysine in an alternative urea cycle (Figure 4). This cycle shares enzymes with the common urea cycle but ornithine, citrulline, argininosuccinate, and arginine are replaced by lysine, homocitrulline, homoargininosuccinate, and homoarginine, respectively.[81-83]

NO is catalytically synthesized by NO synthases (NOS) (Figure 4). Both arginine and homoarginine can serve as substrates, although the latter has a lower efficiency for NO synthesis.[84-86] As described in a previous section, endothelial NO is of major importance for vessel function, organ perfusion, and blood pressure regulation. Inhibition of NOS by endogenously produced monomethylarginine (MMA) and asymmetric dimethylarginine (ADMA) impairs vascular homeostasis (Figure 4).[87]

Proteolysis of proteins containing methylated arginine residues results in release of free MMA, ADMA, and symmetric dimethylarginine (SDMA). In contrast to MMA and ADMA, SDMA has no inhibitory effect on NOS. Because concentrations of MMA are much lower than those of ADMA, the latter is probably the only relevant endogenous NOS inhibitor. Approximately 80% of all ADMA is degraded by dimethylarginine dimethylaminohydrolase (DDAH), the remainder being cleared from the circulation by renal excretion.[88]

It has been shown that ADMA is an independent predictor for CVD and CVD-related mortality.[89] The role of arginine in CVD is less clear, probably because, next to NO production, arginine is involved in many other processes in the human body. Some, but not all, intervention studies showed improved vasodilation, a process generally thought anti-atherogenic, after arginine administration.[90] A complicating factor is that plasma levels of amino acids, that are most easily measured, do not necessarily reflect intracellular concentrations. Cationic amino acid transporters (CAT) can transport cationic amino acids such as (homo)arginine, lysine, and ADMA across the outer cell membrane (Figure 4). Transport by CAT is dependent on local concentrations of cationic amino acids.[91,92] Therefore, high plasma concentrations of homoarginine may reduce the cellular uptake and hence the intracellular concentrations of arginine and vice versa. Because homoarginine is a less efficient substrate for NOS, relatively high homoarginine concentrations may indirectly reduce NO synthesis. However, one study in mice excepted, the role of homoarginine with respect to hypertension and CVD has never been studied.[93]

Study population and measures of outcome

In 1989 a cross-sectional study about glucose metabolism started in Hoorn, The Netherlands. In this study, 3553 residents, aged 50-74 years, were invited. Participants were included if having at least 3 Caucasian grandparents and after written informed consent was obtained. In total 2484 subjects (46% male) were enrolled. In 2000-2001, 1074 subjects were invited again, but only 648 participated in this follow-up examination.[94,95] Major reasons for no show were lack of interest (30%), co-morbidity (23%), high age (7%), or being total non-responders (13%).[95] To have a sufficient number of type 2 diabetes patients for studying glucose metabolism, diabetes patients of the Diabetes Screening Study (Hoorn and direct surroundings)

were added to the follow-up cohort of the Hoorn Study to form the population studied in this thesis.

Blood pressure, flow-mediated dilation (FMD), and nitroglycerin-mediated dilation (NMD) were used as measures of outcome. Both FMD and NMD use ultrasound to measure the diameter of the brachial artery. For FMD, blood flow in the forearm is stopped for 5 minutes by inflating a cuff to supra systolic pressure. Once the cuff is removed, regained blood flow will cause shear stress resulting in increased production of NO and subsequent relaxation of smooth muscle cells. In a healthy artery this process causes the vessel to dilate, but in CVD this mechanism may be impaired. The (relative) change in diameter after release of the cuff is called FMD. In the NMD procedure the NO donor nitroglycerin is administered to induce maximal arterial dilation. FMD is considered to be dependent on both endothelium-dependent (NO production) and endothelium-independent (NO-induced relaxation of smooth muscle cells) processes, whereas NMD reflects endothelium-independent vasodilation. FMD has been shown to be a predictor of both recurrent and incident CVD events.[41,96,97]

Study objectives

-To study the relationships of novel markers of inflammation and nitric oxide signaling with vascular function and blood pressure.

-To investigate whether these relationships are influenced by hyperglycemia and/or oxidative stress.

Outline of the thesis

LDL-cholesterol is a well-known risk marker for CVD. A key event in the development of atherosclerosis is the excessive uptake of oxidized LDL particles by macrophages. We measured oxLDL concentrations in plasma by ELISA. OxLDL concentrations may depend on LDL particle number, which can be estimated by LDL-c and ApoB concentrations. The relation of oxLDL and ratios of oxLDL/LDL-c and oxLDL/ApoB with vascular function have been studied in **chapter 2**.

In **chapter 3** we reviewed the literature on the predictive value of MPO for CVD and mechanisms by which MPO may accelerate atherogenesis. Several types of sample specimens were used in various studies, which hampers a direct comparison between studies, because both coagulation and some anticoagulants potentially induce ex vivo MPO release. To investigate this issue, we compared EDTA-plasma, heparin-plasma and serum as sample material for measurement of MPO in **chapter 4**. We investigated if MPO was associated with endothelium-dependent vasodilation in **chapter 5** and if MPO was associated with blood pressure in **chapter 6A**. Since hyperglycemia has been associated with increased hydrogen peroxide concentrations and hydrogen peroxide is the essential cosubstrate of MPO, we specifically investigated in **chapters 5 and 6A**, whether hyperglycemia and/or oxidative stress enhanced the associations of MPO with vasodilation and blood pressure. In **chapter 6B** we describe that a reduction of MPO activity may contribute to the vasoprotective and blood pressure-lowering effects of melatonin and the antioxidant Pycnogenol. To determine the effects of inflammation and oxidative stress on NO production, associations of MPO and CRP with substrate (arginine) and inhibitor (ADMA) of NOS were studied in **chapter 7.** Since homoarginine shows strong similarity to arginine, it may function as substrate or inhibitor for enzymes that use arginine as substrate, including NOS. Hence, in **chapter 8** we investigated whether homoarginine and/or arginine are associated with blood pressure.

References

1. Mackay J, Mensah G. The atlas of heart disease and stroke. *WHO*. 2004.
2. World Health Organization Fact sheet Cardiovascular diseases No. 317. *WHO*. 2009.
3. Lloyd-Jones D, Adams RJ, Brown TM, Carnethon M, Dai S, De Simone G, Ferguson TB, Ford E, Furie K, Gillespie C, Go A, Greenlund K, Haase N, Hailpern S, Ho PM, Howard V, Kissela B, Kittner S, Lackland D, Lisabeth L, Marelli A, McDermott MM, Meigs J, Mozaffarian D, Mussolino M, Nichol G, Roger VL, Rosamond W, Sacco R, Sorlie P, Stafford R, Thom T, Wasserthiel-Smoller S, Wong ND, Wylie-Rosett J. Heart disease and stroke statistics--2010 update: a report from the American Heart Association. *Circulation*. 2010;121:e1-e170.
4. Stratton IM, Adler AI, Neil HA, Matthews DR, Manley SE, Cull CA, Hadden D, Turner RC, Holman RR. Association of glycaemia with macrovascular and microvascular complications of type 2 diabetes (UKPDS 35): prospective observational study. *BMJ*. 2000;321:405-412.
5. Henry RM, Ferreira I, Kostense PJ, Dekker JM, Nijpels G, Heine RJ, Kamp O, Bouter LM, Stehouwer CD. Type 2 diabetes is associated with impaired endothelium-dependent, flow-mediated dilation, but impaired glucose metabolism is not; The Hoorn Study. *Atherosclerosis*. 2004;174:49-56.
6. Henry RM, Kostense PJ, Spijkerman AM, Dekker JM, Nijpels G, Heine RJ, Kamp O, Westerhof N, Bouter LM, Stehouwer CD. Arterial stiffness increases with deteriorating glucose tolerance status: the Hoorn Study. *Circulation*. 2003;107:2089-2095.
7. Nakanishi N, Shiraishi T, Wada M. Association between fasting glucose and C-reactive protein in a Japanese population: the Minoh study. *Diabetes Res Clin Pract*. 2005;69:88-98.
8. Thorand B, Löwel H, Schneider A, Kolb H, Meisinger C, Fröhlich M, Koenig W. C-reactive protein as a predictor for incident diabetes mellitus among middle-aged men: results from the MONICA Augsburg cohort study, 1984-1998. *Arch Intern Med*. 2003;163:93-99.
9. Jay D, Hitomi H, Griendling KK. Oxidative stress and diabetic cardiovascular complications. *Free Radic Biol Med*. 2006;40:183-192.
10. Zhang L, Zalewski A, Liu Y, Mazurek T, Cowan S, Martin JL, Hofmann SM, Vlassara H, Shi Y. Diabetes-induced oxidative stress and low-grade inflammation in porcine coronary arteries. *Circulation*. 2003;108:472-478.
11. Goff DC, Jr., D'Agostino RB, Jr., Haffner SM, Otvos JD. Insulin resistance and adiposity influence lipoprotein size and subclass concentrations. Results from the Insulin Resistance Atherosclerosis Study. *Metabolism*. 2005;54:264-270.
12. The Emerging Risk Factors Collaboration. Diabetes mellitus, fasting blood glucose concentration, and risk of vascular disease: a collaborative meta-analysis of 102 prospective studies. *Lancet*. 2010;375:2215-2222.
13. Zarbock A, Ley K. Mechanisms and consequences of neutrophil interaction with the endothelium. *Am J Pathol*. 2008;172:1-7.
14. Rao RM, Yang L, Garcia-Cardena G, Luscinskas FW. Endothelial-dependent mechanisms of leukocyte recruitment to the vascular wall. *Circ Res*. 2007;101:234-247.
15. Lusis AJ. Atherosclerosis. *Nature*. 2000;407:233-241.

16. Virchow R. Cellular pathology. As based upon physiological and pathological histology. Lecture XVI--Atheromatous affection of arteries. 1858. *Nutr Rev*. 1989;47:23-25.
17. Konstantinov IE, Mejevoi N, Anichkov NM. Nikolai N. Anichkov and his theory of atherosclerosis. *Tex Heart Inst J*. 2006;33:417-423.
18. Anitschkow N, Chalatow S. Ueber experimentelle Cholesterinsteatose und ihre Bedeutung fur die Entstehung einiger pathologischer Prozesse. *Centralblatt fuer allgemeine Pathologie und pathologische Anatomie*. 1913;24:1-9.
19. Oncley JL, Harvie NR. Lipoproteins--a current perspective of methods and concepts. *Proc Natl Acad Sci U S A*. 1969;64:1107-1118.
20. Havel RJ, Eder HA, Bragdon JH. The distribution and chemical composition of ultracentrifugally separated lipoproteins in human serum. *J Clin Invest*. 1955;34:1345-1353.
21. Ross R, Glomset JA. Atherosclerosis and the arterial smooth muscle cell: Proliferation of smooth muscle is a key event in the genesis of the lesions of atherosclerosis. *Science*. 1973;180:1332-1339.
22. Ross R. Atherosclerosis--an inflammatory disease. *N Engl J Med*. 1999;340:115-126.
23. Williams KJ, Tabas I. The response-to-retention hypothesis of early atherogenesis. *Arterioscler Thromb Vasc Biol*. 1995;15:551-561.
24. Goldstein JL, Ho YK, Basu SK, Brown MS. Binding site on macrophages that mediates uptake and degradation of acetylated low density lipoprotein, producing massive cholesterol deposition. *Proc Natl Acad Sci U S A*. 1979;76:333-337.
25. Steinbrecher UP, Parthasarathy S, Leake DS, Witztum JL, Steinberg D. Modification of low density lipoprotein by endothelial cells involves lipid peroxidation and degradation of low density lipoprotein phospholipids. *Proc Natl Acad Sci U S A*. 1984;81:3883-3887.
26. Morel DW, DiCorleto PE, Chisolm GM. Endothelial and smooth muscle cells alter low density lipoprotein in vitro by free radical oxidation. *Arteriosclerosis*. 1984;4:357-364.
27. Stocker R, Keaney JF, Jr. Role of oxidative modifications in atherosclerosis. *Physiol Rev*. 2004;84:1381-1478.
28. Navab M, Hama SY, Cooke CJ, Anantharamaiah GM, Chaddha M, Jin L, Subbanagounder G, Faull KF, Reddy ST, Miller NE, Fogelman AM. Normal high density lipoprotein inhibits three steps in the formation of mildly oxidized low density lipoprotein: step 1. *J Lipid Res*. 2000;41:1481-1494.
29. Navab M, Hama SY, Anantharamaiah GM, Hassan K, Hough GP, Watson AD, Reddy ST, Sevanian A, Fonarow GC, Fogelman AM. Normal high density lipoprotein inhibits three steps in the formation of mildly oxidized low density lipoprotein: steps 2 and 3. *J Lipid Res*. 2000;41:1495-1508.
30. Kontush A, Chapman MJ. Antiatherogenic small, dense HDL--guardian angel of the arterial wall? *Nat Clin Pract Cardiovasc Med*. 2006;3:144-153.
31. Marsche G, Furtmüller PG, Obinger C, Sattler W, Malle E. Hypochlorite-modified high-density lipoprotein acts as a sink for myeloperoxidase in vitro. *Cardiovasc Res*. 2008;79:187-194.

32. De Souza JA, Vindis C, Hansel B, Nègre-Salvayre A, Therond P, Serrano CV, Jr., Chantepie S, Salvayre R, Bruckert E, Chapman MJ, Kontush A. Metabolic syndrome features small, apolipoprotein A-I-poor, triglyceride-rich HDL3 particles with defective anti-apoptotic activity. *Atherosclerosis*. 2007;197:84-94.
33. Zheng L, Nukuna B, Brennan ML, Sun M, Goormastic M, Settle M, Schmitt D, Fu X, Thomson L, Fox PL, Ischiropoulos H, Smith JD, Kinter M, Hazen SL. Apolipoprotein A-I is a selective target for myeloperoxidase-catalyzed oxidation and functional impairment in subjects with cardiovascular disease. *J Clin Invest*. 2004;114:529-541.
34. Luscher TF, Barton M. Biology of the endothelium. *Clin Cardiol*. 1997;20:II-10.
35. Moncada S, Higgs EA. The discovery of nitric oxide and its role in vascular biology. *Br J Pharmacol*. 2006;147:S193-S201.
36. Hermo R, Mier C, Mazzotta M, Tsuji M, Kimura S, Gugliucci A. Circulating levels of nitrated apolipoprotein A-I are increased in type 2 diabetic patients. *Clin Chem Lab Med*. 2005;43:601-606.
37. Förstermann U, Münzel T. Endothelial nitric oxide synthase in vascular disease: from marvel to menace. *Circulation*. 2006;113:1708-1714.
38. Vasan RS. Biomarkers of cardiovascular disease: molecular basis and practical considerations. *Circulation*. 2006;113:2335-2362.
39. Rifai N, Gerszten RE. Biomarker discovery and validation. *Clin Chem*. 2006;52:1635-1637.
40. Gerszten RE, Wang TJ. The search for new cardiovascular biomarkers. *Nature*. 2008;451:949-952.
41. Katz SD, Hryniewicz K, Hriljac I, Balidemaj K, Dimayuga C, Hudaihed A, Yasskiy A. Vascular endothelial dysfunction and mortality risk in patients with chronic heart failure. *Circulation*. 2005;111:310-314.
42. Lippincott MF, Carlow A, Desai A, Blum A, Rodrigo M, Patibandla S, Zalos G, Smith K, Schenke WH, Csako G, Waclawiw MA, Cannon RO, III. Relation of endothelial function to cardiovascular risk in women with sedentary occupations and without known cardiovascular disease. *Am J Cardiol*. 2008;102:348-352.
43. Wilson PW, D'Agostino RB, Levy D, Belanger AM, Silbershatz H, Kannel WB. Prediction of coronary heart disease using risk factor categories. *Circulation*. 1998;97:1837-1847.
44. Stamler J, Vaccaro O, Neaton JD, Wentworth D. Diabetes, other risk factors, and 12-yr cardiovascular mortality for men screened in the Multiple Risk Factor Intervention Trial. *Diabetes Care*. 1993;16:434-444.
45. Sorkin JD, Muller DC, Fleg JL, Andres R. The relation of fasting and 2-h postchallenge plasma glucose concentrations to mortality: data from the Baltimore Longitudinal Study of Aging with a critical review of the literature. *Diabetes Care*. 2005;28:2626-2632.
46. Pepys MB, Hirschfield GM. C-reactive protein: a critical update. *J Clin Invest*. 2003;111:1805-1812.

47. Chait A, Han CY, Oram JF, Heinecke JW. Thematic review series: The immune system and atherogenesis. Lipoprotein-associated inflammatory proteins: markers or mediators of cardiovascular disease? *J Lipid Res*. 2005;46:389-403.
48. Muntner P, He J, Chen J, Fonseca V, Whelton PK. Prevalence of non-traditional cardiovascular disease risk factors among persons with impaired fasting glucose, impaired glucose tolerance, diabetes, and the metabolic syndrome: analysis of the Third National Health and Nutrition Examination Survey (NHANES III). *Ann Epidemiol*. 2004;14:686-695.
49. Ridker PM, Buring JE, Shih J, Matias M, Hennekens CH. Prospective study of C-reactive protein and the risk of future cardiovascular events among apparently healthy women. *Circulation*. 1998;98:731-733.
50. Haider DG, Leuchten N, Schaller G, Gouya G, Kolodjaschna J, Schmetterer L, Kapiotis S, Wolzt M. C-reactive protein is expressed and secreted by peripheral blood mononuclear cells. *Clin Exp Immunol*. 2006;146:533-539.
51. Kardys I, Knetsch AM, Bleumink GS, Deckers JW, Hofman A, Stricker BH, Witteman JC. C-reactive protein and risk of heart failure. The Rotterdam Study. *Am Heart J*. 2006;152:514-520.
52. Kivimäki M, Lawlor DA, Smith GD, Kumari M, Donald A, Britton A, Casas JP, Shah T, Brunner E, Timpson NJ, Halcox JP, Miller MA, Humphries SE, Deanfield J, Marmot MG, Hingorani AD. Does high C-reactive protein concentration increase atherosclerosis? The Whitehall II Study. *PLoS One*. 2008;3:e3013.
53. Elliott P, Chambers JC, Zhang W, Clarke R, Hopewell JC, Peden JF, Erdmann J, Braund P, Engert JC, Bennett D, Coin L, Ashby D, Tzoulaki I, Brown IJ, Mt-Isa S, McCarthy MI, Peltonen L, Freimer NB, Farrall M, Ruokonen A, Hamsten A, Lim N, Froguel P, Waterworth DM, Vollenweider P, Waeber G, Jarvelin MR, Mooser V, Scott J, Hall AS, Schunkert H, Anand SS, Collins R, Samani NJ, Watkins H, Kooner JS. Genetic Loci associated with C-reactive protein levels and risk of coronary heart disease. *JAMA*. 2009;302:37-48.
54. Itabe H, Takeshima E, Iwasaki H, Kimura J, Yoshida Y, Imanaka T, Takano T. A monoclonal antibody against oxidized lipoprotein recognizes foam cells in atherosclerotic lesions. Complex formation of oxidized phosphatidylcholines and polypeptides. *J Biol Chem*. 1994;269:15274-15279.
55. Penny WF, Ben Yehuda O, Kuroe K, Long J, Bond A, Bhargava V, Peterson JF, McDaniel M, Juliano J, Witztum JL, Ross J, Jr., Peterson KL. Improvement of coronary artery endothelial dysfunction with lipid-lowering therapy: heterogeneity of segmental response and correlation with plasma-oxidized low density lipoprotein. *J Am Coll Cardiol*. 2001;37:766-774.
56. Mäkimattila S, Liu ML, Vakkilainen J, Schlenzka A, Lahdenperä S, Syvänne M, Mantysaari M, Summanen P, Bergholm R, Taskinen MR, Yki-Jarvinen H. Impaired endothelium-dependent vasodilation in type 2 diabetes. Relation to LDL size, oxidized LDL, and antioxidants. *Diabetes Care*. 1999;22:973-981.

57. Holvoet P, Macy E, Landeloos M, Jones D, Nancy JS, Van de Werf F, Tracy RP. Analytical performance and diagnostic accuracy of immunometric assays for the measurement of circulating oxidized LDL. *Clin Chem*. 2006;52:760-764.
58. Ashton EL, Best JD, Ball MJ. Effects of monounsaturated enriched sunflower oil on CHD risk factors including LDL size and copper-induced LDL oxidation. *J Am Coll Nutr*. 2001;20:320-326.
59. Aviram M, Fuhrman B. Wine flavonoids protect against LDL oxidation and atherosclerosis. *Ann N Y Acad Sci*. 2002;957:146-161.
60. Holvoet P, Theilmeier G, Shivalkar B, Flameng W, Collen D. LDL hypercholesterolemia is associated with accumulation of oxidized LDL, atherosclerotic plaque growth, and compensatory vessel enlargement in coronary arteries of miniature pigs. *Arterioscler Thromb Vasc Biol*. 1998;18:415-422.
61. Holvoet P, Vanhaecke J, Janssens S, Van de Werf F, Collen D. Oxidized LDL and malondialdehyde-modified LDL in patients with acute coronary syndromes and stable coronary artery disease. *Circulation*. 1998;98:1487-1494.
62. Klebanoff SJ. Myeloperoxidase: friend and foe. *J Leukoc Biol*. 2005;77:598-625.
63. Klebanoff SJ. Myeloperoxidase-halide-hydrogen peroxide antibacterial system. *J Bacteriol*. 1968;95:2131-2138.
64. Lakshmi VM, Nauseef WM, Zenser TV. Myeloperoxidase potentiates nitric oxide-mediated nitrosation. *J Biol Chem*. 2005;280:1746-1753.
65. Exner M, Hermann M, Hofbauer R, Hartmann B, Kapiotis S, Gmeiner B. Thiocyanate catalyzes myeloperoxidase-initiated lipid oxidation in LDL. *Free Radic Biol Med*. 2004;37:146-155.
66. Klebanoff SJ, Waltersdorph AM, Rosen H. Antimicrobial activity of myeloperoxidase. *Methods Enzymol*. 1984;105:399-403.
67. Baldus S, Eiserich JP, Mani A, Castro L, Figueroa M, Chumley P, Ma W, Tousson A, White CR, Bullard DC, Brennan ML, Lusis AJ, Moore KP, Freeman BA. Endothelial transcytosis of myeloperoxidase confers specificity to vascular ECM proteins as targets of tyrosine nitration. *J Clin Invest*. 2001;108:1759-1770.
68. Sugiyama S, Okada Y, Sukhova GK, Virmani R, Heinecke JW, Libby P. Macrophage myeloperoxidase regulation by granulocyte macrophage colony-stimulating factor in human atherosclerosis and implications in acute coronary syndromes. *Am J Pathol*. 2001;158:879-891.
69. Singh U, Devaraj S, Jialal I. C-reactive protein stimulates myeloperoxidase release from polymorphonuclear cells and monocytes: implications for acute coronary syndromes. *Clin Chem*. 2009;55:361-364.
70. Baldus S, Rudolph V, Roiss M, Ito WD, Rudolph TK, Eiserich JP, Sydow K, Lau D, Szocs K, Klinke A, Kubala L, Berglund L, Schrepfer S, Deuse T, Haddad M, Risius T, Klemm H, Reichenspurner HC, Meinertz T, Heitzer T. Heparins increase endothelial nitric oxide

bioavailability by liberating vessel-immobilized myeloperoxidase. *Circulation*. 2006;113:1871-1878.

71. Leculier C, Couprie N, Adeleine P, Leitienne P, Francina A, Richard M. The effects of high molecular weight- and low molecular weight-heparins on superoxide ion production and degranulation by human polymorphonuclear leukocytes. *Thromb Res*. 1993;69:519-531.
72. Li G, Keenan AC, Young JC, Hall MJ, Pamuklar Z, Ohman EM, Steinhubl SR, Smyth SS. Effects of unfractionated heparin and glycoprotein IIb/IIIa antagonists versus bivalirdin on myeloperoxidase release from neutrophils. *Arterioscler Thromb Vasc Biol*. 2007;27:1850-1856.
73. Boudjeltia KZ, Legssyer I, Van Antwerpen P, Kisoka RL, Babar S, Moguilevsky N, Delree P, Ducobu J, Remacle C, Vanhaeverbeek M, Brohee D. Triggering of inflammatory response by myeloperoxidase-oxidized LDL. *Biochem Cell Biol*. 2006;84:805-812.
74. Wu Z, Wagner MA, Zheng L, Parks JS, Shy JM, III, Smith JD, Gogonea V, Hazen SL. The refined structure of nascent HDL reveals a key functional domain for particle maturation and dysfunction. *Nat Struct Mol Biol*. 2007;14:861-868.
75. McCall MR, Carr AC, Forte TM, Frei B. Ldl modified by hypochlorous acid is a potent inhibitor of lecithin-cholesterol acyltransferase activity. *Arterioscler Thromb Vasc Biol*. 2001;21:1040-1045.
76. Zheng L, Settle M, Brubaker G, Schmitt D, Hazen SL, Smith JD, Kinter M. Localization of nitration and chlorination sites on apolipoprotein A-I catalyzed by myeloperoxidase in human atheroma and associated oxidative impairment in ABCA1-dependent cholesterol efflux from macrophages. *J Biol Chem*. 2005;280:38-47.
77. Bergt C, Pennathur S, Fu X, Byun J, O'Brien K, McDonald TO, Singh P, Anantharamaiah GM, Chait A, Brunzell J, Geary RL, Oram JF, Heinecke JW. The myeloperoxidase product hypochlorous acid oxidizes HDL in the human artery wall and impairs ABCA1-dependent cholesterol transport. *Proc Natl Acad Sci U S A*. 2004;101:13032-13037.
78. Sugiyama S, Kugiyama K, Aikawa M, Nakamura S, Ogawa H, Libby P. Hypochlorous acid, a macrophage product, induces endothelial apoptosis and tissue factor expression: involvement of myeloperoxidase-mediated oxidant in plaque erosion and thrombogenesis. *Arterioscler Thromb Vasc Biol*. 2004;24:1309-1314.
79. Srivenugopal KS, Adiga PR. Partial purification and properties of a transamidinase from Lathyrus sativus seedlings. Involvement in homoarginine metabolism and amine interconversions. *Biochem J*. 1980;189:553-560.
80. Da Silva RP, Nissim I, Brosnan ME, Brosnan JT. Creatine synthesis: hepatic metabolism of guanidinoacetate and creatine in the rat in vitro and in vivo. *Am J Physiol Endocrinol Metab*. 2009;296:E256-E261.
81. Ryan WL, Wells IC. Homocitrulline and homoarginine synthesis from lysine. *Science*. 1964;144:1122-1127.
82. Ryan WL, Barak AJ, Johnson RJ. Lysine, homocitrulline, and homoarginine metabolism by the isolated perfused rat liver. *Arch Biochem Biophys*. 1968;123:294-297.

83. Ryan WL, Johnson RJ, Dimari S. Homoarginine synthesis by rat kidney. *Arch Biochem Biophys*. 1969;131:521-526.
84. Moali C, Boucher JL, Sari MA, Stuehr DJ, Mansuy D. Substrate specificity of NO synthases: detailed comparison of L-arginine, homo-L-arginine, their N omega-hydroxy derivatives, and N omega-hydroxynor-L-arginine. *Biochemistry*. 1998;37:10453-10460.
85. Henningsson R, Lundquist I. Arginine-induced insulin release is decreased and glucagon increased in parallel with islet NO production. *Am J Physiol*. 1998;275:E500-E506.
86. Inoue Y, Bode BP, Beck DJ, Li AP, Bland KI, Souba WW. Arginine transport in human liver. Characterization and effects of nitric oxide synthase inhibitors. *Ann Surg*. 1993;218:350-362.
87. Leiper J, Nandi M, Torondel B, Murray-Rust J, Malaki M, O'Hara B, Rossiter S, Anthony S, Madhani M, Selwood D, Smith C, Wojciak-Stothard B, Rudiger A, Stidwill R, McDonald NQ, Vallance P. Disruption of methylarginine metabolism impairs vascular homeostasis. *Nat Med*. 2007;13:198-203.
88. Achan V, Broadhead M, Malaki M, Whitley G, Leiper J, MacAllister R, Vallance P. Asymmetric dimethylarginine causes hypertension and cardiac dysfunction in humans and is actively metabolized by dimethylarginine dimethylaminohydrolase. *Arterioscler Thromb Vasc Biol*. 2003;23:1455-1459.
89. Siroen MP, Teerlink T, Nijveldt RJ, Prins HA, Richir MC, Van Leeuwen PA. The clinical significance of asymmetric dimethylarginine. *Annu Rev Nutr*. 2006;26:203-228.
90. Bode-Böger SM, Scalera F, Ignarro LJ. The L-arginine paradox: Importance of the L-arginine/asymmetrical dimethylarginine ratio. *Pharmacol Ther*. 2007;114:295-306.
91. Teerlink T, Luo Z, Palm F, Wilcox CS. Cellular ADMA: Regulation and action. *Pharmacol Res*. 2009;60:448-460.
92. White MF, Gazzola GC, Christensen HN. Cationic amino acid transport into cultured animal cells. I. Influx into cultured human fibroblasts. *J Biol Chem*. 1982;257:4443-4449.
93. Chen PY, Sanders PW. Role of nitric oxide synthesis in salt-sensitive hypertension in Dahl/Rapp rats. *Hypertension*. 1993;22:812-818.
94. Mooy JM, Grootenhuis PA, De Vries H, Valkenburg HA, Bouter LM, Kostense PJ, Heine RJ. Prevalence and determinants of glucose intolerance in a Dutch caucasian population. The Hoorn Study. *Diabetes Care*. 1995;18:1270-1273.
95. Bos G, Scheffer PG, Vieira D, Dekker JM, Nijpels G, Diamant M, Teerlink T, Stehouwer CD, Bouter LM, Heine RJ, Jansen H. The relationship of lipoprotein lipase activity and LDL size is dependent on glucose metabolism in an elderly population: the Hoorn Study. *Diabetes Care*. 2004;27:796-798.
96. Meyer B, Mörtl D, Strecker K, Hülsmann M, Kulemann V, Neunteufl T, Pacher R, Berger R. Flow-mediated vasodilation predicts outcome in patients with chronic heart failure: comparison with B-type natriuretic peptide. *J Am Coll Cardiol*. 2005;46:1011-1018.
97. Yeboah J, Folsom AR, Burke GL, Johnson C, Polak JF, Post W, Lima JA, Crouse JR, Herrington DM. Predictive value of brachial flow-mediated dilation for incident cardiovascular

events in a population-based study: the multi-ethnic study of atherosclerosis. *Circulation*. 2009;120:502-509.

Chapter 2

Circulating oxidized LDL: determinants and association with brachial flow-mediated dilation

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Abstract

Circulating oxidized LDL (oxLDL) levels are strongly correlated to LDL-cholesterol (LDL-c) and apolipoprotein-B100 (apoB100), making it difficult to disentangle their independent contributions to cardiovascular risk. We explored the determinants of oxLDL and the relation between oxLDL and flow-mediated dilation (FMD) of the brachial artery to investigate whether the oxLDL/LDL-c and oxLDL/apoB100 ratios are more informative than the separate variables. FMD of the brachial artery and plasma concentrations of oxLDL, LDL-cholesterol, and apoB100 were measured in 624 men and women (age range 50 to 87 years), participating in a population-based cohort study. OxLDL was strongly correlated with apoB100 (r = 0.82, P < 0.001) and LDL-c (r = 0.67, P < 0.001). Other major independent determinants of oxLDL were sex, HDL-cholesterol, and LDL particle size. LDL-c and apoB100 concentrations were not significantly associated with FMD. After adjustment for age, sex, glucose tolerance status, and Framingham risk score, the oxLDL/apoB100 ratio was negatively related to FMD (P = 0.017). This association was weaker for the oxLDL/ LDL-c ratio (P = 0.062) and absent for oxLDL level (P = 0.27). In contrast to oxLDL, the oxLDL/apoB100 ratio, and to a lesser extent the oxLDL/LDL-c ratio, are related to a functional measure of atherosclerosis. Therefore, correction of oxLDL for LDL particle number may improve the clinical usefulness of oxLDL measurement.

Introduction

Atherosclerosis is considered a process involving the interplay of inflammation and oxidative stress. Oxidation of LDL and the subsequent uptake by macrophages in the vascular wall are important steps in the development of atherosclerosis.[1] A small part of the oxidized LDL (oxLDL) particles escapes uptake by macrophages and returns to the blood stream or may leak from atherosclerotic plaques. Thus, measuring circulating levels of oxLDL may contribute to the estimation of cardiovascular disease (CVD) risk. In support of this notion, concentrations of oxLDL were elevated in patients with stable and unstable angina and acute myocardial infarction.[2] In apparently healthy middle-aged men, oxLDL was found to be a strong predictor for acute coronary heart disease events.[3]

The concentration of oxLDL depends not only on the degree of oxidative stress, but also on the amount of substrate for oxidation (i.e., the number of LDL particles). Indeed, oxLDL levels were consistently found to be strongly correlated to LDL-cholesterol (LDL-c) and apolipoprotein-B100 (apoB100) in several studies,[3-8] making it difficult to disentangle their separate contributions to CVD. Therefore, to establish the additional value of oxLDL in the assessment of CVD risk, it may be necessary to account for the number of LDL particles. LDL-c and apoB100 have been used for this purpose, but their merits have never been compared.

Impaired flow-mediated dilation (FMD) of the brachial artery has been shown to predict future cardiovascular events.[9,10] Previous studies have investigated the relationship between oxLDL and FMD in specific patient populations,[11-13] but this relation has not yet been evaluated in a large-population-based sample. We have used FMD data from the Hoorn Study, a community-based cohort study among elderly men and women, to assess the suitability of the oxLDL/LDL-c and oxLDL/apoB100 ratios to correct for LDL particle number. We have explored the determinants of oxLDL and the relations between oxLDL and the oxLDL/LDL-c and oxLDL/apoB100 ratios with FMD to establish whether these ratios provide information beyond the separate variables.

Materials and methods

Subjects

The present investigation was conducted in the 2000 follow-up examination of the Hoorn Study[14] and the Hoorn Screening Study[15], both of which were population-based studies in Caucasians. From the study population (n = 822), we excluded persons using lipid-lowering medication (n = 134) and with missing data on primary variables of interest (n = 64), leaving 624 individuals (300 men and 324 women), of whom 233 had normal glucose metabolism, 159 impaired glucose metabolism, and 232 type 2 diabetes according to WHO-99 criteria.[16] The local ethics committee approved the study and all participants gave their written informed consent.

Plasma lipids and apoB100

LDL-c was directly determined by the “N-geneous” assay (GenZyme, Cambridge, MA). Intra- and interassay coefficients of variation (CV) were 0.7% and 2.7%, respectively. Triglyceride concentrations up to 13.5 mmol/L do not interfere with this assay. Total and HDL-cholesterol (HDL-c) and triglycerides were measured by standard enzymatic methods (Roche, Mannheim, Germany). ApoB100 concentrations were determined nephelometrically using an “Immage 800” immunochemistry system (Beckman Coulter Inc., Fullerton, CA) with intra- and interassay CVof 4.9% and 5.1%, respectively.

In vivo oxLDL

A competitive ELISA (Mercodia, Uppsala, Sweden) was used to determine oxLDL concentrations in EDTA-plasma. The 4E6-monoclonal antibody of the assay is directed against apoB100 with at least 60 lysines substituted by aldehydes[2] and is highly specific for oxLDL.[17] Intra- and interassay CV were 6.7% and 7.0%, respectively. To estimate the extent of LDL oxidation, the ratio of oxLDL to LDL-c and the ratio of oxLDL to apoB100 were calculated. These estimates were expressed as U/mmol LDL-c and U/g apoB100, respectively.

LDL particle size and in vitro oxidizability

LDL was isolated by ultracentrifugation between densities 1.019 and 1.063 kg/L. LDL particle size was determined by highperformance gel-filtration chromatography as described previously, using thyroglobulin (17.0 nm) and fibrinogen (22.2 nm) as calibrators.[18] Intra- and inter-assay CV were 0.1% and 0.2%, respectively.

The susceptibility of LDL to in vitro oxidation was determined by measurement of conjugated dienes, after addition of copper ions as pro-oxidant, as previously described.[19] The resistance of LDL to oxidation was expressed as lag time (min). The intra- and interassay CV were 1.6% and 3.6%, respectively.

Glucose metabolism parameters

HbA1c was analyzed by ion exchange high-performance liquid chromatography (Bio-Rad, Veenendaal, The Netherlands). Fasting glucose was measured enzymatically (Roche, Mannheim, Germany) and fasting insulin with a double-antibody radioimmunoassay (Linco Research, St. Louis).

Measurement of endothelium-dependent and endothelium-independent dilation

Ultrasound examination of the right brachial artery was performed according to the guidelines of the International Brachial Artery Reactivity Task Force.[20] Baseline diameter, blood flow (peak systolic velocity), FMD, and nitroglycerin-mediated dilation (NMD) were determined by one single observer (RMAH) as previously described.[21] The intraobserver CV were 4.3% for diameter, 14.7% for FMD, and 10.3% for NMD.[21] Of the 624 participants, qualitatively satisfactory ultrasound examinations were obtained in 484 individuals. Poor definition of the arterial wall due to obesity and inability to remain motionless due to muscoskeletal disorders were the main reasons for missing ultrasound data.[21]

Other measurements

Plasma C-reactive protein (CRP) was measured with a highly sensitive in-house sandwich enzyme-linked immunosorbent assay.[22] Data on smoking habits and alcohol consumption were obtained by a questionnaire. Microalbuminuria was defined as urinary albumin-creatinine ratio ≥ 2 mg/mmol. Prior CVD was defined as Minnesota Code 1.1–1.3, 4.1–4.3, 5.1–5.3, or 7.1 on the electrocardiogram or coronary bypass operation or angioplasty, or an ankle-brachial blood pressure index < 0.9 in either leg, peripheral arterial bypass, or amputation for atherosclerotic disease. The Framingham risk score was calculated.[23]

Statistical analyses

Data are presented as mean (SD) or median (interquartile range). Skewed variables were log-transformed prior to trend analyses and multiple linear regression analyses. In regression models for FMD, we considered age, sex, glucose tolerance status, baseline diameter, and the increase in peak systolic velocity as standard correction variables. In models for NMD, age, sex, glucose tolerance status, and baseline diameter were included. $P < 0.05$ was considered to indicate statistical significance. All analyses were performed using SPSS software, version 15 (SPSS Inc., Chicago, IL).

Results

Subject characteristics

The characteristics of the subjects by tertiles of circulating oxLDL levels are shown in Table 1. With increasing oxLDL concentrations, the percentage of women, apoB100, LDL-c, triglycerides, total cholesterol, HbA1c, fasting glucose, insulin, CRP, body mass index, and waist circumference increased significantly, while a decreasing trend was observed for HDL-c and LDL-size. Current smoking status tended to a significant positive relationship with oxLDL. There were no significant linear trends for age, LDL in vitro oxidation, serum albumin, blood pressure, alcohol consumption, microalbuminuria, and prior CVD across the oxLDL tertiles.

Table 1. Subject characteristics in tertiles of oxidized LDL (oxLDL).

	Unit	Overall	1st tertile	2nd tertile	3rd tertile	$P_{\text{for trend}}$
OxLDL, range	U/L		<57.6	57.6-71.3	>71.3	
Number		624	208	208	208	
Age	years	69.0 (7.3)	69.1 (7.6)	69.7 (7.4)	68.3 (6.6)	0.22
Female sex	%	52	46	53	57	0.019
ApoB100	g/L	1.04 (0.23)	0.85 (0.15)	1.03 (0.15)	1.26 (0.19)	<0.001
LDL-c	mmol/L	3.8 (0.9)	3.1 (0.7)	3.7 (0.6)	4.4 (0.8)	<0.001
HDL-c	mmol/L	1.39 (0.40)	1.50 (0.43)	1.42 (0.40)	1.25 (0.34)	<0.001
Triglycerides[a]	mmol/L	1.3 (1.0-1.8)	1.1 (0.8-1.4)	1.3 (1.0-1.7)	1.8 (1.3-2.4)	<0.001
Total cholesterol	mmol/L	5.8 (1.0)	5.1 (0.8)	5.8 (0.8)	6.5 (0.9)	<0.001
LDL particle size	nm	21.49 (0.45)	21.60 (0.38)	21.55 (0.42)	21.32 (0.51)	<0.001
LDL oxidizability	min	72.0 (9.7)	72.7 (9.9)	71.4 (9.0)	71.9 (10.2)	0.41
Glucose metabolism						
- Impaired	%	25.5	26.9	20.2	29.3	0.57
- Type 2 diabetes	%	37.2	34.6	38.0	38.9	0.36
HbA1c	%	6.1 (0.8)	6.0 (0.7)	6.0 (0.6)	6.2 (0.9)	0.005
Fasting glucose[a]	mmol/L	6.0 (5.5-6.9)	6.0 (5.5-6.7)	6.0 (5.5-7.0)	6.2 (5.6-7.2)	0.018
Insulin[a]	pmol/L	60 (42-88)	55 (40-78)	61 (43-91)	65 (45-94)	0.002
C-reactive protein[a]	mg/L	2.3 (1.1-4.8)	1.8 (1.0-3.4)	2.3 (1.0-4.5)	2.7 (1.4-6.5)	<0.001
Serum albumin	g/L	42 (40-43)	42 (40-43)	41 (40-43)	41 (40-43)	0.20
BMI	kg/m^2	27.6 (4.3)	26.9 (4.3)	27.6 (4.1)	28.3 (4.4)	<0.001
Waist circumference	cm	96.1 (12.5)	94.4 (12.3)	95.6 (12.2)	98.2 (12.7)	0.002
DBP	mmHg	83 (11)	83 (10)	83 (11)	83 (11)	0.47
SBP	mmHg	142 (21)	141 (18)	143 (23)	143 (22)	0.38
Alcohol intake						
<1 g/day	%	29	29	26	31	0.63
1-40 g/day	%	64	62	66	63	0.78
>40 g/day	%	8	9	7	6	0.18
Current smoking	%	17	13	16	20	0.050
Micro-albuminuria	%	15	14	15	15	0.87
Prior CVD	%	44	41	44	48	0.13

Values are displayed either as means (SD), medians (interquartile range), or percentages. [a]Variables were log-transformed prior to linear trend analysis.

Adjustment of oxLDL for particle number

The trends with oxLDL shown in Table 1 were confirmed by linear regression analysis after adjustment for age and sex (Table 2). The associations of oxLDL with apoB100 and LDL-c were particularly strong, reflecting the fact that the total amount of LDL is an important determinant of oxLDL concentration (Table 2 and Figure 1). To correct oxLDL levels for LDL particle number, the oxLDL/LDL-c and oxLDL/apoB100 ratios were calculated.

Table 2. Age and sex adjusted associations with oxidized LDL (oxLDL), and the oxLDL/LDL-c and oxLDL/apoB100 ratios

		OxLDL		OxLDL/ LDL-c		OxLDL/ apoB100	
Variable	Units or categories	st. β	*P*	st. β	*P*	st. β	*P*
Age	Years	-0.085	0.033	-0.082	0.042	-0.031	0.45
Sex	female vs. male	0.110	0.006	-0.054	0.18	-0.037	0.36
OxLDL	U/L			0.323	<0.001	0.322	<0.001
ApoB100	g/L	0.815	<0.001	-0.033	0.42	-0.275	<0.001
LDL-c	mmol/L	0.671	<0.001	-0.443	<0.001	-0.222	<0.001
HDL-c	mmol/L	-0.389	<0.001	-0.348	<0.001	-0.220	<0.001
Triglycerides[a]	mmol/L	0.541	<0.001	0.390	<0.001	0.156	<0.001
Total cholesterol	mmol/L	0.715	<0.001	-0.276	<0.001	-0.229	<0.001
LDL particle size	Nm	-0.367	<0.001	-0.503	<0.001	-0.240	<0.001
LDL oxidizability (lagtime)	min.	-0.003	0.95	0.081	0.044	0.064	0.11
Glucose metabolism	impaired vs. normal	0.102	0.022	0.062	0.16	0.060	0.18
	diabetes vs. normal	0.107	0.016	0.265	<0.001	0.172	<0.001
HbA1c	%	0.147	<0.001	0.202	<0.001	0.123	0.002
Fasting glucose[a]	mmol/L	0.141	<0.001	0.243	<0.001	0.141	<0.001
Insulin[a]	pmol/L	0.147	<0.001	0.288	<0.001	0.166	<0.001
C-reactive protein[a]	mg/L	0.148	<0.001	0.236	<0.001	0.127	<0.002
Serum albumin	g/L	-0.041	0.31	-0.059	0.15	-0.170	<0.001
BMI	kg/m^2	0.143	<0.001	0.139	<0.001	0.118	0.003
Waist circumference	Cm	0.181	<0.001	0.185	<0.001	0.145	<0.001
Diastolic blood pressure	mmHg	0.045	0.26	0.064	0.11	0.027	0.50
Systolic blood pressure	mmHg	0.046	0.26	0.072	0.078	0.012	0.76
Alcohol intake	1-40 g/day	-0.008	0.85	-0.134	0.003	-0.142	0.002
	>40 g/day	-0.003	0.95	0.040	0.38	-0.026	0.57
Current smoking	yes vs. no	0.077	0.055	0.051	0.20	0.046	0.25

[a]Variables were log-transformed prior to analyses. Regression coefficients are presented as standardized beta.

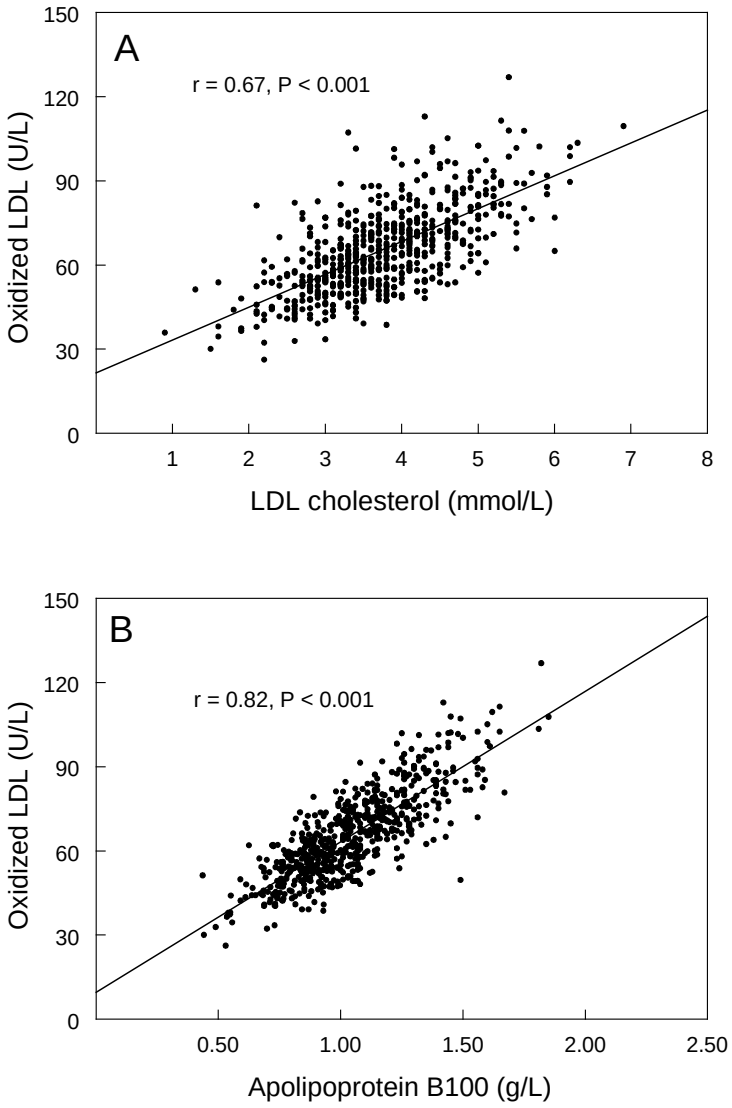

Figure 1. The association between oxidized LDL and LDL cholesterol (A) and the association between oxidized LDL (oxLDL) and apolipoprotein-B100 (apoB100) (B).

Determinants of oxLDL

The variables associated with oxLDL/LDL-c and oxLDL/apoB100 ratios in linear regression analyses were identical to the variables associated with oxLDL, with a few exceptions (Table 2). The positive association with female sex was lost, and moderate alcohol intake (1–40 g/day) was negatively associated with both ratios. Furthermore, the oxLDL/apoB100 ratio was negatively associated with serum albumin. To establish which variables other than LDL-c and apoB100 were independent determinants of (adjusted) oxLDL, we explored multivariate linear

regression models with oxLDL, the oxLDL/LDL-c ratio, and the oxLDL/apoB100 ratio as dependent variables (Table 3). In all models, an identical set of potential predictor variables, excluding apoB100 and LDL-c, was used. Determinants other than age and sex were selected because of significant univariate associations and/or biological plausibility. Categories of glucose metabolism were used as a proxy for all variables associated with glucose metabolism. Because triglycerides were strongly associated with LDL particle size ($r = 0.67$; $P<0.001$), only the latter was included in the models. LDL particle size was the only independent variable shared by all three models. Female sex and low HDL-cholesterol were additional major determinants of oxLDL. Additional independent determinants of the oxLDL/LDL-c and oxLDL/apoB100 ratios were high CRP and low serum albumin, respectively. Furthermore, a weak negative association between moderate alcohol intake and the oxLDL/apoB100 ratio was observed. Age, categories of glucose metabolism, waist circumference, and current smoking did not significantly contribute to any of the models. The full models explained 20%, 28%, and 11% of the variability of oxLDL, oxLDL/LDL-c, and oxLDL/apoB100, respectively. If LDL-c or apoB100 were added as independent variables to the regression model for oxLDL, the explained variance increased to 63 and 71%, respectively.

Table 3. Multivariable linear regression models for oxidized LDL (oxLDL), the oxLDL/LDL-c ratio, and the oxLDL/apoB100 ratio

		OxLDL		OxLDL/LDL-c		oxLDL/apoB100	
Independent variable	Units of increase or categories	st. Beta	*P*-value	st. Beta	*P*-value	st. Beta	*P*-value
Age	Years	-0.060	0.12	-0.052	0.16	-0.042	0.31
Sex	female vs. Male	**0.30**	**<0.001**	0.059	0.16	0.028	0.54
Glucose metabolism	impaired vs. Normal	0.032	0.45	-0.029	0.47	0.016	0.73
	diabetes vs. Normal	-0.078	0.10	0.061	0.18	0.083	0.10
Waist circumference	Cm	0.066	0.14	-0.019	0.66	0.046	0.33
LDL particle diameter	Nm	**-0.25**	**<0.001**	**-0.43**	**<0.001**	**-0.16**	**0.003**
HDL-cholesterol	mmol/L	**-0.24**	**<0.001**	-0.039	0.43	-0.072	0.20
C-reactive protein	mg/L, log transformed	0.024	0.58	**0.11**	**0.006**	-0.010	0.82
Serum albumin	g/L	-0.020	0.61	-0.059	0.12	**-0.18**	**<0.001**
Current smoking	yes vs. no	0.047	0.21	0.030	0.40	0.027	0.50
Alcohol intake	1-40 g/day	0.054	0.20	-0.052	0.19	**-0.088**	**0.047**
	>40 g/day	0.017	0.70	0.036	0.40	-0.004	0.93
R^2 model		0.20		0.28		0.11	

Regression coefficients are presented as standardized beta; independent variables significantly contributing to the models are printed in bold

OxLDL and vascular function

Neither LDL-c nor apoB100 were associated with FMD in unadjusted analysis or after adjustment for the standard variables age, sex, baseline diameter, glucose tolerance status, and increase in peak systolic velocity (all *P*>0.5). Mean FMD values did not significantly differ between subjects with levels of oxLDL below or above the median value (Table 4 and Figure 2A). In contrast, if the study population was dichotomized according to the oxLDL/LDL-c ratio or oxLDL/apoB100 ratio, the mean FMD was approximately 15% lower in subjects with values of these ratios above the median (Figure 2B, C).

Table 4. Brachial artery characteristics according to levels of oxidized LDL (oxLDL)

OxLDL	Below median <64 U/L	Above median >64 U/L	*P*-value
N	244	240	
Diameter (µm)			
Baseline	4687 (768)	4599 (702)	0.20
After FMD	4866 (789)	4768 (709)	0.18
After NMD	5131 (803)	5030 (707)	0.18
Absolute change in diameter (µm)			
After FMD	177 (171)	171 (145)	0.70
After NMD	453 (214)	448 (217)	0.62
Percentage change in diameter (%)			
After FMD	3.96 (3.74)	3.87 (3.36)	0.99
After NMD	10.17 (5.77)	10.07 (5.87)	0.85
Peak systolic velocity (cm/s)			
Baseline	58 (13)	57 (12)	0.40
After reactive hyperemia	105 (25)	105 (26)	0.68
% Increase	84 (39)	88 (48)	0.62

Results are expressed as mean (SD). FMD, flow-mediated dilation; NMD, nitroglycerin-mediated dilation. *P*-values were calculated by Mann-Whitney analyses.

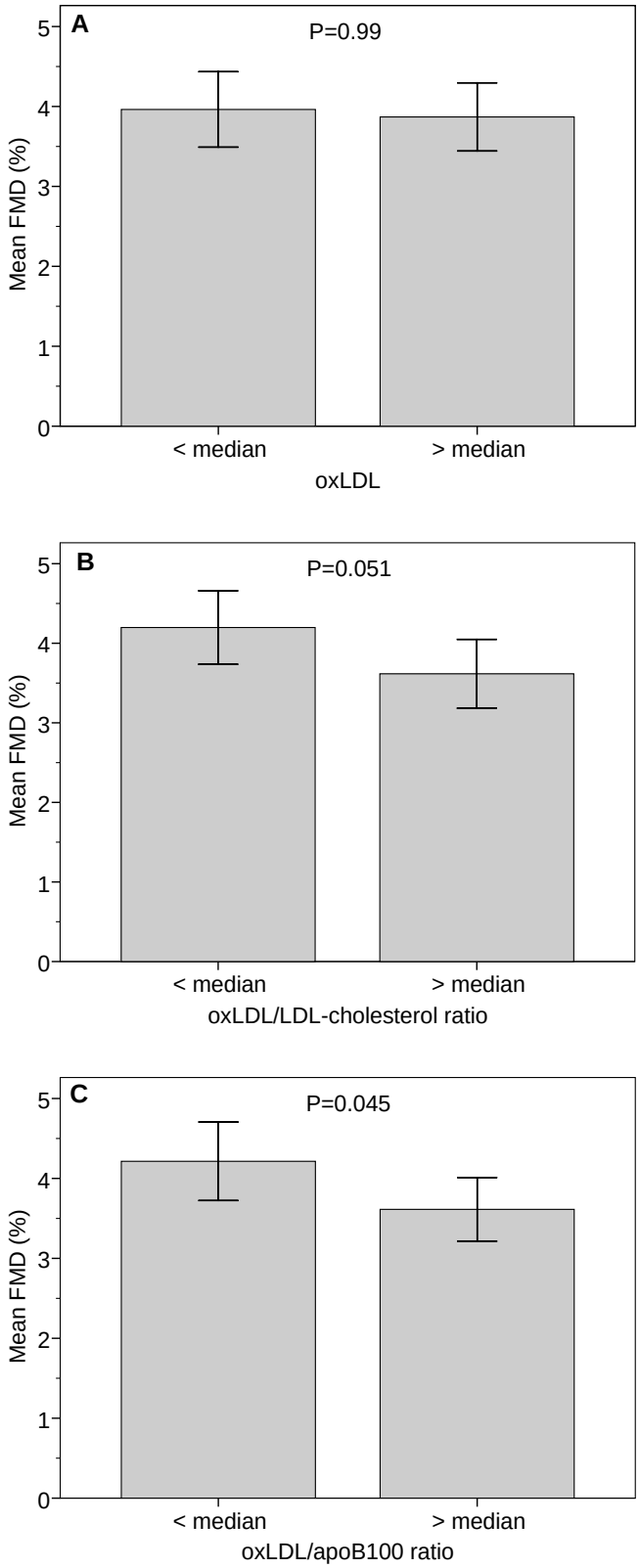

Figure 2. Flow-mediated dilation (FMD) of the brachial artery according to low (below the median) or high (above the median) values of oxLDL (A), the oxLDL/LDL-cholesterol (LDL-c) ratio (B), or the oxLDL/ apoB100 ratio (C). FMD data are presented as means with 95% confidence intervals. Significance of differences was tested by Mann-Whitney test.

To investigate whether the relationship between (adjusted) oxLDL and FMD was independent of traditional risk factors, several multiple linear regression models were explored (Table 5). After adjustment for standard variables, oxLDL was negatively related to FMD, but this association lost significance upon adjustment for prior CVD or Framingham risk score. The oxLDL/LDL-c ratio was negatively related to FMD with borderline significance, in the standard model and after additional adjustment. In contrast, the negative association between the oxLDL/apoB100 ratio and FMD remained highly significant after adjustment for prior CVD, Framingham risk score, microalbuminuria, and waist circumference. Mean NMD values did not significantly differ between subjects with levels of oxLDL below or above the median value (Table 4). In linear regression models, neither oxLDL nor the oxLDL/LDL-c and oxLDL/apoB100 ratios were associated with NMD (all $P > 0.3$), and the negative associations between these variables and FMD were not attenuated after adjustment for NMD (Table 5, models 5 and 7).

Table 5. Multivariable linear regression models with flow-mediated dilation as dependent variable and oxLDL, oxLDL/LDL-c ratio, or oxLDL/apoB100 ratio as predictor variables

Independent variable	**Beta (95% confidence interval)**	***P*-value**
OxLDL, per 1 SD (15.4 U/L)		
Standard model	**-12.8 (-25.3 to -0.2)**	**0.048**
Model 1: standard + prior CVD	-12.1 (-24.7 to 0.5)	0.060
Model 2: standard + Framingham risk score	-8.0 (-22.3 to 6.3)	0.27
Model 3: standard + (micro)albuminuria	**-13.2 (-25.7 to -0.6)**	**0.040**
Model 4: standard + waist circumference	-11.1 (-23.8 to 1.7)	0.088
Model 5: standard + nitroglycerin-mediated dilation	**-11.8 (-23.2 to -0.6)**	**0.039**
Model 6: fully adjusted	-7.2 (-21.4 to 6.9)	0.32
Model 7: fully adjusted + nitroglycerin-mediated dilation	-8.1 (-20.7 to 4.6)	0.21
OxLDL/LDL-c ratio, per 1 SD (3.8 U/mmol)		
Standard model	-11.9 (-24.3 to 0.5)	0.060
Model 1: standard + prior CVD	-10.6 (-23.1 to 1.8)	0.093
Model 2: standard + Framingham risk score	-11.8 (-24.2 to 0.6)	0.062
Model 3: standard + (micro)albuminuria	-10.4 (-22.8 to 2.1)	0.10
Model 4: standard + waist circumference	-10.6 (-23.1 to 1.9)	0.097
Model 5: standard + nitroglycerin-mediated dilation	**-18.0 (-29.1 to -6.9)**	**0.002**
Model 6: fully adjusted	-8.3 (-20.8 to 4.2)	0.19
Model 7: fully adjusted + nitroglycerin-mediated dilation	**-14.6 (-25.8 to -3.4)**	**0.011**
OxLDL/apoB100 ratio, per 1 SD (9.1 U/g)		
Standard model	**-14.9 (-27.2 to -2.6)**	**0.018**
Model 1: standard + prior CVD	**-14.3 (-26.6 to -2.0)**	**0.023**
Model 2: standard + Framingham risk score	**-14.9 (-27.2 to -2.7)**	**0.017**
Model 3: standard + (micro)albuminuria	**-14.0 (-26.3 to -1.7)**	**0.025**
Model 4: standard + waist circumference	**-13.8 (-26.1 to -1.5)**	**0.028**
Model 5: standard + nitroglycerin-mediated dilation	**-16.9 (-27.9 to -5.9)**	**0.003**
Model 6: fully adjusted	**-12.8 (-25.1 to -0.5)**	**0.040**
Model 7: fully adjusted + nitroglycerin-mediated dilation	**-14.8 (-25.7 to -3.8)**	**0.008**

Standard model: determinant under consideration + age, sex, glucose tolerance status, baseline diameter, and increase in peak systolic velocity. Fully adjusted model: standard model + prior CVD, Framingham risk score, (micro)albuminuria, and waist circumference. Regression coefficients are expressed as absolute change in diameter (in µm) per 1 SD increase of the independent variable to facilitate direct comparison. Significant associations are printed in bold.

Discussion

The present study shows that the total amount of LDL, either expressed as LDL-c or as apoB100 level, is by far the strongest determinant of oxLDL levels. In contrast to LDL-c, apoB100 and oxLDL concentrations; the oxLDL/LDL-c ratio; and, more prominently, the oxLDL/apoB100 ratio, independent of traditional risk factors, were related to endothelium-dependent dilation of the brachial artery. Correction of oxLDL for LDL particle number may thus improve the clinical usefulness of oxLDL measurement.

OxLDL concentrations not only depend on the level of oxidative stress, but also on the amount of substrate for oxidation (i.e., the total amount of LDL or the numberof LDL particles). Because it is difficult to exactly determine the number of LDL particles, both LDL-c and apoB100 concentrations have been used as estimates.[3-8] Adjustment of oxLDL for either LDL-c or apoB100 may be essential for a correct interpretation of the data, particularly in intervention studies. For example, Van Tits et al.[4] observed no effect of statin use on the oxLDL/apoB100 ratio, although a significant decrease in unadjusted oxLDL concentration was observed. Thus, adjustments of oxLDL by LDL-c or apoB100 have been described in several studies, but, to our knowledge, a direct comparison of both adjustments has been performed in only one study.[8] Both approaches may have some limitations. LDL-c is often estimated with the Friedewald formula, which may lead to reduced accuracy and precision. However, even if LDL-c is measured directly, as we did, it is not a perfect measure for LDL particle number, because LDL particles are heterogeneous and vary in cholesterol content.[24] Due to the fact that each LDL particle contains exactly 1 apoB100 molecule, the apoB100 concentration may be a better estimator for the LDL particle number. Still, up to approximately 10% of apoB100 molcules is present in VLDL particles,[25] resulting in a slight overestimation of LDL particle number. As illustrated in Figure 1, the scatter in the relation between oxLDL and LDL-c was higher compared with the relation between oxLDL and apoB100. In agreement with this, the age and sex adjusted association between oxLDL and apoB100 was stronger than with LDL-c (Table 2). Altogether, apoB100 seems more suitable than LDL-c for adjustment of oxLDL.

In sex- and age-corrected analyses, many clinical and biochemical variables were significantly associated with oxLDL, and the oxLDL/LDL-c and oxLDL/apoB100

ratios (Table 2). In multivariate linear regression models with oxLDL, the oxLDL/LDL-c ratio, or the oxLDL/apoB100 ratio as dependent variable, most of these associations lost significance, and only a few independent determinants were identified. LDL particle diameter was by far the strongest predictor in all three models, in agreement with the observation that small LDL particles are more prone to oxidation than larger LDL particles.[26] HDL-c was a negative determinant of oxLDL, consistent with the antioxidative properties of HDL.[27] Likewise, the negative association between serum albumin and the oxLDL/apoB100 ratio may reflect the antioxidative properties of albumin. This association may, however, also reflect the relation between low albumin levels and lowgrade inflammation.[28] The strong positive association between CRP, a marker of inflammation,[29] and the oxLDL/LDL-c ratio confirms that inflammation plays a role in the process of LDL oxidation.

Type 2 diabetes and variables related to glucose metabolism were positively related to oxLDL (Table 2), consistent with a role of LDL oxidation in the elevated cardiovascular morbidity and mortality in diabetes.[30,31] However, diabetes was not an independent determinant in the multivariate regression models. This may be explained by the fact that diabetic patients generally have low HDL-c levels and small LDL particles, both of which were associated with high oxLDL levels.

Alcohol consumption is related to CVD. Excessive alcohol consumption is proatherogenic, while moderate consumption of alcohol may have an anti-atherogenic effect. Paradoxically, Schroder et al.[32] observed a positive relation between oxLDL concentrations and moderate alcohol consumption. In the present study, moderate alcohol consumption was negatively associated with the oxLDL/LDL-c and oxLDL/apoB100 ratios. However, after full adjustment, this association was only borderline significant in the model for oxLDL/apoB100. Likewise, current smoking was positively associated with oxLDL with borderline significance in univariate analysis, but did not contribute to the multivariate models.

To investigate whether adjustment of oxLDL levels for LDL particle number may be relevant in clinical studies, we explored the impact of adjustment on the relation between oxLDL and FMD. Neither LDL-c nor apoB100 were significantly related to FMD, and oxLDL showed a borderline significant negative association. In contrast, the oxLDL/apoB100 ratio showed a significant, negative association with FMD, even after adjustment for other risk factors or NMD. These results strongly suggest that this ratio yields more information than the separate variables. Therefore,

using the oxLDL/apoB100 ratio rather than the oxLDL level is not equivalent to statistical adjustment of the relation between oxLDL and FMD for apoB100.

Although our study was not designed to unravel the mechanisms linking elevated levels of oxLDL and FMD, our data allow drawing some tentative conclusions. Formation of oxLDL is promoted by oxidative stress, but oxLDL itself has also been identified as a potent stimulus for vascular free radical formation.[33] This may lead to a vicious circle, making it virtually impossible to separate cause and effect. The inverse relation between oxLDL levels and FMD may indicate that oxidative stress is a common antecedent of diminished vasodilation and LDL oxidation. A second potential mechanism is that oxLDL itself, by inflicting damage to the endothelium, has a direct adverse effect on vasodilation. It should be noted that both mechanisms are not mutually exclusive but may act in concert. Our data, showing that the level of oxidative stress, as indicated by the oxLDL/apoB100 ratio, is a stronger determinant of FMD than the absolute concentration of oxLDL, supports a major role for the first mechanism.

The lack of a significant association between oxLDL/ apoB100 and NMD suggests that the effect is mainly endothelium dependent. This is corroborated by the fact that the strength of the association between oxLDL/apoB100 and FMD was not attenuated by adjustment for NMD. Nitric oxide is the most important endothelium-derived vasodilator in conduit arteries, and it is well known that scavenging of nitric oxide by reactive oxygen species, in particular superoxide, reduces the bioavailability of nitric oxide in the vascular wall. Overall, the results of this study support the notion that the oxLDL/apoB100 ratio is an indicator of vascular production of reactive oxygen species, which by scavenging nitric oxide, limit vasodilation. However, our data do not fully rule out the possibility that oxLDL itself also has an adverse effect on vasodilation.

Our study had some limitations. First, the study population was limited to elderly Caucasians, and therefore the results may be different in other ethnic and age groups. Second, exclusion of subjects using lipid-lowering medication probably has led to selection of subjects with a low cardiovascular risk profile. This may have resulted in an underestimation of the strength of associations between oxLDL and CVD risk factors and vascular function. Third, the assay used to measure oxLDL specifically detects epitopes of oxidized apoB100. However, oxLDL is not a single homogeneous entity, and assays detecting epitopes on other constituents of oxLDL

may yield different results. A major strength of our study is that LDL-c was measured directly and not estimated by Friedewald formula. In addition, we had a large array of clinical and biochemical variables at our disposal to adjust for possible confounding.

Our data confirm that LDL-c and more prominently the number of LDL particles, estimated as apoB100, is a strong determinant of circulating oxLDL levels. The oxLDL/LDL-c and oxLDL/apoB100 ratios, which express the level of LDL oxidation independent of particle number, were shown to provide information beyond oxLDL, LDL-c, and apoB100, as evidenced by the independent association of these ratios with endothelium-dependent dilation of the brachial artery.

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References

1. Lusis AJ. Atherosclerosis. *Nature*. 2000;407:233-241.
2. Holvoet P, Vanhaecke J, Janssens S, Van de Werf F, Collen D. Oxidized LDL and malondialdehyde-modified LDL in patients with acute coronary syndromes and stable coronary artery disease. *Circulation*. 1998;98:1487-1494.
3. Meisinger C, Baumert J, Khuseyinova N, Loewel H, Koenig W. Plasma oxidized low-density lipoprotein, a strong predictor for acute coronary heart disease events in apparently healthy, middle-aged men from the general population. *Circulation*. 2005;112:651-657.
4. Van Tits LJ, Van Himbergen TM, Lemmers HL, De Graaf J, Stalenhoef AF. Proportion of oxidized LDL relative to plasma apolipoprotein B does not change during statin therapy in patients with heterozygous familial hypercholesterolemia. *Atherosclerosis*. 2006;185:307-312.
5. Metso S, Loimaala A, Mercuri MF, Nenonen A, Vuori I, Oja P, Bond MG, Laine S, Rontu R, Lehtimaki T. Circulating oxidized low-density lipoprotein and common carotid artery intima-media thickness in a random sample of middle-aged men. *J Biomed Sci*. 2004;11:356-361.
6. Cesari M, Kritchevsky SB, Nicklas BJ, Penninx BW, Holvoet P, Koh-Banerjee P, Cummings SR, Harris TB, Newman AB, Pahor M. Lipoprotein peroxidation and mobility limitation: results from the health, aging, and body composition study. *Arch Intern Med*. 2005;165:2148-2154.
7. Scheffer PG, Henry RM, Wever EJ, Van Rooij GJ, Bos G, Heine RJ, Dekker JM, Diamant M, Stehouwer CD, Nijpels G, Blankenstein MA, Teerlink T. LDL oxidative modifications in well- or moderately controlled type 2 diabetes. *Diabetes Metab Res Rev*. 2004;20:298-304.
8. Tsuzura S, Ikeda Y, Suehiro T, Ota K, Osaki F, Arii K, Kumon Y, Hashimoto K. Correlation of plasma oxidized low-density lipoprotein levels to vascular complications and human serum paraoxonase in patients with type 2 diabetes. *Metabolism*. 2004;53:297-302.
9. Katz SD, Hryniewicz K, Hriljac I, Balidemaj K, Dimayuga C, Hudaihed A, Yasskiy A. Vascular endothelial dysfunction and mortality risk in patients with chronic heart failure. *Circulation*. 2005;111:310-314.
10. Gokce N, Keaney JF, Jr., Hunter LM, Watkins MT, Menzoian JO, Vita JA. Risk stratification for postoperative cardiovascular events via noninvasive assessment of endothelial function: a prospective study. *Circulation*. 2002;105:1567-1572.
11. Paniagua JA, Lopez-Miranda J, Perez-Martinez P, Marin C, Vida JM, Fuentes F, Fernandez de la Puebla RA, Perez-Jimenez F. Oxidized-LDL levels are changed during short-term serum glucose variations and lowered with statin treatment in early Type 2 diabetes: a study of endothelial function and microalbuminuria. *Diabet Med*. 2005;22:1647-1656.
12. Jarvisalo MJ, Lehtimaki T, Raitakari OT. Determinants of arterial nitrate-mediated dilatation in children: role of oxidized low-density lipoprotein, endothelial function, and carotid intima-media thickness. *Circulation*. 2004;109:2885-2889.
13. Matsuoka H. Endothelial dysfunction associated with oxidative stress in human. *Diabetes Res Clin Pract*. 2001;54 Suppl 2:S65-S72.

14. Mooy JM, Grootenhuis PA, De Vries H, Valkenburg HA, Bouter LM, Kostense PJ, Heine RJ. Prevalence and determinants of glucose intolerance in a Dutch caucasian population. The Hoorn Study. *Diabetes Care*. 1995;18:1270-1273.
15. Spijkerman AM, Adriaanse MC, Dekker JM, Nijpels G, Stehouwer CD, Bouter LM, Heine RJ. Diabetic patients detected by population-based stepwise screening already have a diabetic cardiovascular risk profile. *Diabetes Care*. 2002;25:1784-1789.
16. Alberti KG, Zimmet PZ. Definition, diagnosis and classification of diabetes mellitus and its complications. Part 1: diagnosis and classification of diabetes mellitus provisional report of a WHO consultation. *Diabet Med*. 1998;15:539-553.
17. Holvoet P, Mertens A, Verhamme P, Bogaerts K, Beyens G, Verhaeghe R, Collen D, Muls E, Van de Werf F. Circulating oxidized LDL is a useful marker for identifying patients with coronary artery disease. *Arterioscler Thromb Vasc Biol*. 2001;21:844-848.
18. Scheffer PG, Bakker SJ, Heine RJ, Teerlink T. Measurement of low-density lipoprotein particle size by high-performance gel-filtration chromatography. *Clin Chem*. 1997;43:1904-1912.
19. Scheffer PG, Bakker SJ, Popp-Snijders C, Heine RJ, Schutgens RB, Teerlink T. Composition of LDL as determinant of its susceptibility to in vitro oxidation in patients with well-controlled type 2 diabetes. *Diabetes Metab Res Rev*. 2001;17:459-466.
20. Corretti MC, Anderson TJ, Benjamin EJ, Celermajer D, Charbonneau F, Creager MA, Deanfield J, Drexler H, Gerhard-Herman M, Herrington D, Vallance P, Vita J, Vogel R. Guidelines for the ultrasound assessment of endothelial-dependent flow-mediated vasodilation of the brachial artery: a report of the International Brachial Artery Reactivity Task Force. *J Am Coll Cardiol*. 2002;39:257-265.
21. Henry RM, Ferreira I, Kostense PJ, Dekker JM, Nijpels G, Heine RJ, Kamp O, Bouter LM, Stehouwer CD. Type 2 diabetes is associated with impaired endothelium-dependent, flow-mediated dilation, but impaired glucose metabolism is not; The Hoorn Study. *Atherosclerosis*. 2004;174:49-56.
22. Grooteman MP, Gritters M, Wauters IM, Schalkwijk CG, Stam F, Twisk J, Ter Wee PM, Nube MJ. Patient characteristics rather than the type of dialyser predict the variability of endothelial derived surface molecules in chronic haemodialysis patients. *Nephrol Dial Transplant*. 2005;20:2751-2758.
23. Wilson PW, D'Agostino RB, Levy D, Belanger AM, Silbershatz H, Kannel WB. Prediction of coronary heart disease using risk factor categories. *Circulation*. 1998;97:1837-1847.
24. Teerlink T, Scheffer PG, Bakker SJ, Heine RJ. Combined data from LDL composition and size measurement are compatible with a discoid particle shape. *J Lipid Res*. 2004;45:954-966.
25. Schonfeld G, Lees RS, George PK, Pfleger B. Assay of total plasma apolipoprotein B concentration in human subjects. *J Clin Invest*. 1974;53:1458-1467.
26. De Graaf J, Hak-Lemmers HL, Hectors MP, Demacker PN, Hendriks JC, Stalenhoef AF. Enhanced susceptibility to in vitro oxidation of the dense low density lipoprotein subfraction in healthy subjects. *Arterioscler Thromb*. 1991;11:298-306.

27. Navab M, Ananthramaiah GM, Reddy ST, Van Lenten BJ, Ansell BJ, Fonarow GC, Vahabzadeh K, Hama S, Hough G, Kamranpour N, Berliner JA, Lusis AJ, Fogelman AM. The oxidation hypothesis of atherogenesis: the role of oxidized phospholipids and HDL. *J Lipid Res*. 2004;45:993-1007.
28. Kaysen GA. Association between inflammation and malnutrition as risk factors of cardiovascular disease. *Blood Purif*. 2006;24:51-55.
29. Ridker PM. High-sensitivity C-reactive protein: potential adjunct for global risk assessment in the primary prevention of cardiovascular disease. *Circulation*. 2001;103:1813-1818.
30. Stocker R, Keaney JF, Jr. Role of oxidative modifications in atherosclerosis. *Physiol Rev*. 2004;84:1381-1478.
31. Holvoet P. Oxidized LDL and coronary heart disease. *Acta Cardiol*. 2004;59:479-484.
32. Schroder H, Marrugat J, Fito M, Weinbrenner T, Covas MI. Alcohol consumption is directly associated with circulating oxidized low-density lipoprotein. *Free Radic Biol Med*. 2006;40:1474-1481.
33. Galle J, Hansen-Hagge T, Wanner C, Seibold S. Impact of oxidized low density lipoprotein on vascular cells. *Atherosclerosis.* 2006;185:219-226.

Chapter 3

Myeloperoxidase: a useful biomarker for cardiovascular disease risk stratification?

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Abstract

Background: Inflammation and oxidative stress are associated with atherosclerosis. Myeloperoxidase (MPO) is linked to both inflammation and oxidative stress by its location in leukocytes and its role in catalyzing the formation of oxidizing agents. Recent evidence suggests that MPO activity precipitates atherogenesis. Measurement of MPO in plasma may therefore contribute to cardiovascular disease (CVD) risk stratification.

Content: Cross-sectional studies, case-control studies, and prospective-cohort studies investigating the relation between MPO and CVD have been evaluated. Differences in study populations, sample materials, sample handling, and assays were ascertained. Potential causal mechanisms linking MPO to accelerated atherosclerosis are discussed here. A majority of studies indicate that measurement of MPO in plasma was associated with improved CVD risk stratification above and beyond risk stratification results obtained with markers used in routine clinical practice. However, comparison of these epidemiological studies with regard to MPO and outcome is hampered because the reported MPO concentration depends on the assay method, sampling material, and preanalytical and analytical procedures. The link between MPO and CVD can, at least partly, be explained by MPO-dependent oxidation of LDL and HDL, subsequently leading to cholesterol accumulation in the arterial wall. Furthermore, MPO may reduce the bioavailability of nitric oxide, resulting in endothelial dysfunction. Finally, MPO destabilizes atherosclerotic plaques.

Summary: Increasing evidence suggests that MPO is causally linked to atherosclerosis and its measurement may improve CVD risk estimation. Before MPO can be used in routine clinical practice, however, standardization of sampling and laboratory procedures is needed.

Introduction

Cardiovascular disease (CVD) is the leading cause of death in Western societies. CVD mortality and morbidity are promoted by major CVD risk factors, such as hyperlipidemia, hypertension, and smoking. The sequence of events leading to CVD includes endothelial dysfunction, atherosclerotic plaque formation, and rupture.[1] Inflammation has been implicated in all these stages in the evolution of atherosclerotic plaques.[1] Moreover, oxidative stress is currently considered a key event in CVD development.[2]

Myeloperoxidase (MPO) is an enzyme linked to both inflammation and oxidative stress. It is abundantly expressed in the azurophilic granules of most leukocyte subspecies, including neutrophils and monocytes.[3] MPO is released by leukocytes in a state of inflammation and catalyzes the formation of several reactive species, including hypochlorous acid, and thus has a role in host defense against microorganisms.[3] Paradoxically, MPO has been implicated in initiation and propagation of atherosclerosis. In the past few years, evidence emerging from epidemiological studies has shown that higher concentrations of MPO are associated with an increased CVD risk, independent of classical CVD risk factors.[4-15] However, comparison of these epidemiological studies with regard to MPO and CVD outcome is hampered by the fact that various assay methods and sampling procedures have been applied. The differences that are attributable to the use of various methods may have important implications for the interpretation of the results of these studies.

This review presents epidemiological evidence for MPO as a relevant biomarker with respect to CVD outcome, reviews the pathophysiological mechanisms linking MPO to CVD, and addresses preanalytical and analytical issues in measurement of MPO.

Myeloperoxidase and cardiovascular disease: epidemiological studies

A number of epidemiological studies have been reported that address the association of MPO with CVD and markers of atherosclerosis in a wide range of patients populations, including patients with established atherosclerosis and patients with an acute presentation with chest pain (Table 1).

Established atherosclerosis

The first epidemiological report assessing the association between MPO and CVD was a case-control study published by Zhang and coworkers in 2001.[15] The authors studied the relation of MPO, expressed either as mass per milligram of neutrophil protein or as MPO mass per milliliter of blood, with coronary artery disease (CAD) risk in a case-control design with 158 patients with established CAD and 175 controls. CAD patients had significantly higher concentrations of MPO compared to the controls, and it was demonstrated by multivariate logistic regression analysis adjusted for traditional CVD risk factors that MPO was associated with an 11.9-fold (95% CI, 5.5–25.5, upper vs lower quartile) increased risk for CAD for leukocyte MPO, and a 20.4-fold (95% CI, 8.9–47.7, upper vs lower quartile) increased risk for CAD for blood MPO. In a cross-sectional study by Duzguncinar et al., MPO was increased in patients with CAD and correlated to the extent and severity of atherosclerosis of the coronary vessels.[16] Meuwese and coworkers studied the association of MPO with CAD in 1138 patients with CAD and 2237 controls.[11] These investigators demonstrated that MPO (upper vs lower quartile) was related to CAD after adjustment for traditional risk factors [odds ratio (OR), 1.36 (95% CI, 1.07–1.73)]. In a case-control study in 680 patients, 382 patients with stable CAD and 194 controls with normal coronary angiograms, MPO was higher in patients with CAD compared to controls.[14] In addition, MPO concentrations were found to correlate with the presence of CAD [OR, 2.08 (95% CI, 1.54 –2.81)]. Mocatta and coworkers studied the 5-year all-cause mortality rate in 512 patients with established myocardial infarction.[12] Patients with MPO values above the median had an almost 2-fold increased risk [hazard ratio (HR) 1.81 (95% CI, 1.07–3.05)] of all-cause mortality compared to those with MPO values below the median. The predictive value of MPO for mortality and myocardial infarction was studied by Baldus et al. in a group of 1090 patients with acute coronary syndromes during a 6-month follow-up period.[4] Although MPO did not correlate with established markers of CVD risk and inflammation, including troponin-T, soluble CD40 ligand, and C-reactive protein (CRP), patients with increased MPO (upper vs lower tertile) had a 2.25-fold (95% CI, 1.32–3.82) increased risk of adverse events, defined as reinfarction or death. In a multivariate model, MPO was the strongest independent predictor of CVD outcome. In 38 patients with ST-segment myocardial infarction presenting with cardiogenic shock and treated

with percutaneous coronary interventions, baseline MPO was an independent predictor of inhospital mortality [OR, 3.9 (95% CI: 1.8–7.5)], after adjustment for clinical, laboratory, and angiographic variables.[8] Brevetti et al. studied the predictive value of MPO vs CRP for fatal and nonfatal CVD events in 156 patients with peripheral artery disease.[6] Despite the relatively low number of events (n = 17) during 6 months of follow-up, the authors demonstrated that MPO was a strong predictor for adverse events [HR 6.80 (95% CI, 1.20 –38.69)], whereas CRP was not [HR 0.88 (95% CI, 0.60 –1.29)]. Exner et al. studied the progression of stenosis of the internal carotid artery in 1019 asymptomatic CAD patients with a follow-up of 7.5 months.[9] Patients with progressive stenosis had significantly higher baseline MPO concentrations compared to patients with stable disease. Interestingly, the relation between MPO and progression of stenosis was modified by HDL cholesterol level. An MPO concentration above the median was associated with a 2.6-fold increased risk (95% CI, 1.4–4.8) of disease progression, but only in patients with HDL cholesterol concentrations below the median. Altogether, the results of these studies indicate that MPO may be a valuable novel marker for CVD events. However, one should keep in mind that publication bias due to unpublished negative findings cannot be ruled out. Stefanescu and coworkers found no independent association between MPO and all-cause mortality in 382 patients with stable CAD during 3.5 years of follow-up.[17] In contrast to the above-mentioned studies, all of which had reported significant associations between MPO and CVD, 1 case-control study in HIV patients showed no significant independent association of MPO with CVD events.[18] Another study, in patients undergoing elective coronary angiography, showed no significant differences in MPO concentrations for those with proven stable CAD compared to those without proven CAD.[19] A possible explanation for this negative finding might be the lower risk in stable vs unstable CAD. Indeed, cardiovascular events are thought to be more likely in unstable CVD.[20] These observations may thus indicate that MPO is a particularly useful biomarker in high-risk populations.

Table 1. Overview of prospective and case-control studies assessing the association of myeloperoxidase with CVD

First Author year (Ref#)	Sample type	Study Population	N, cases (controls)	Men, %	Age, years	Follow-up, months	Outcome	Risk, 95%CI	Comparison
Apple 2007[21]	heparin plasma	patients with ACS	457	57% (no event) 48% (event)	57±16 (no event) 62±18 (event)	4	all-cause mortality	0.9 (0.4-2.1)	above vs below 99th percentile
Baldus 2003[4]	serum	patients with ACS	1090	71 (low MPO) 69 (high MPO)	61.4±10.5 (low MPO) 62.5±10.4 (high MPO)	6	MI or mortality	2.25 (1.32-3.82)	high vs low MPO
Brennan 2003[5]	plasma	patients with chest pain	142 (462)	55 (no MI) 70 (MI)	61.4 ± 13.8 (no MI) 66.5 ± 12.8 (MI)	1 and 6	MI	4.7 (2.9-7.7) 4.7 (2.8-7.7)	4th vs 1st quartile MPO
Brevetti 2008[6]	serum	patients with PAD	156	77	67.1±8.2	6-40	CVD events	6.80 (1.20-38.7)	high vs low MPO
Cavusoglu 2007[7]	plasma	patients with ACS	193	100	65.0± 9.3 (low MPO) 64.7±10.8 (high MPO)	24	MI	1.60 (1.09-2.36)	per 1 SD of log(MPO)
Dominguez 2008[8]	serum	patients STEMI and CS	38	78 (survivors) 65 (non-survivors)	66±10 (survivors) 75±12 (non-survivors)	NA	in-hospital mortality	3.9 (1.8-7.5)	MPO, continuous
El-Bejjani 2008[18]	plasma	HIV infected adults	62 (62)	94	46.0 (40-52) 45.5 (39-51)	12	CVD events	NA	cases vs. controls
Exner 2006[9]	serum	asymptomatic CAD patients	1019	62	69 (range: 61-76)	7.5	progression of ICA stenosis	2.57 (1.39-4.75)	above vs below MPO median
Khan 2007[10]	EDTA-plasma	patients with STEMI	384 (257)	21 (1st quartile) 15 (4th quartile)	61.8±12.3 (1st quartile) 67.6±11.9 (4th quartile)	>1	death and non-fatal MI	6.91 (1.79-26.73)	above vs below median log(MPO)
Meuwese 2007[11]	serum	healthy individuals	1138 (2237)	63 (controls) 64 (cases)	65.3±7.7 (controls) 65.5±7.8 (cases)	96	CAD	1.36 (1.07-1.73)	log(MPO), continuous
Mocatta 2007[12]	EDTA-plasma	patients with MI	512	80	61.7±11.0	60	all-cause mortality	1.81 (1.07-3.05)	above vs below MPO median
Morrow 2008[13]	plasma	patients with ACS	1524	68 (below median) 66 (above median)	61 (52-69) 61 (53-70)	1	non-fatal MI or hospitalization	2.10 (1.36-3.23)	log(MPO), continuous
Ndrepepa 2008[14]	EDTA-plasma	patients with stable CAD	680 (194)	42 (controls) 73 (cases)	58.7 (controls) 74.5 (cases)	NA	ACS	2.08 (1.54-2.81)	MPO, continuous
Stefanescu 2008[17]	EDTA-plasma	patients with stable CAD	382	46 (1st tertiles) 72 (3rd tertile)	64.7 (1st tertile) 68.4 (3rd tertile)	42	all-cause mortality	1.06 (0.71-1.59)	high vs low and intermediate MPO
Zhang 2001[15]	leukocytes /blood	patients with and without established CAD	158 (175)	58 (controls) 80 (cases)	55±10 (controls) 64±13 (cases)	NA	NA	11.9 (5.5-25.5)/ 20.4 (8.9-47.2)	cases vs controls

Abbreviations: ACS, acute coronary syndrome; CAD, coronary artery disease; CI, confidence interval; CS, cardiogenic shock; ICA, internal carotid artery; MI, myocardial infarction; MPO, Myeloperoxidase; N, number of subjects; NA, not applicable; PAD, peripheral artery disease; Ref#, reference number; STEMI, ST-segment elevation MI.

Acute presentation with chest pain

In a cross-sectional study by Esporcatte et al., an MPO concentration higher than 100 pmol/L had a diagnostic sensitivity of 92% and specificity of 40% as a marker for identifying patients with an acute myocardial infarction (AMI) presenting with acute chest pain and non–ST elevation electrocardiogram findings. In this study, AMI was defined by troponin I concentration of >1.0 µg/L.[22] In contrast, a study by Apple et al. found no additional diagnostic value of MPO (99th percentile) compared to troponin I in patients with clinically diagnosed acute coronary syndrome (ACS).[23] Although MPO and troponin I showed similar diagnostic sensitivities, the diagnostic specificity of MPO was considerably lower than troponin I. Furthermore, a previous study by Apple et al.[21] showed that in patients presenting with symptoms suggestive of ACS, increased troponin I was significantly associated with all-cause mortality, whereas increased MPO (99th percentile) was not. Morrow and coworkers studied the predictive value of MPO, soluble CD40 ligand, troponin I, and CRP in 1524 patients with ACS in a tirofiban intervention trial for survival within 180 days. Patients with increased MPO concentrations (above median) were at higher risk for nonfatal MI or rehospitalization for ACS at 30 days. Furthermore, MPO was associated with recurrent ischemic events, after adjustment for CRP, troponin I, soluble CD40 ligand, and other major CVD risk factors.[13] The clinical value of MPO to predict AMI and adverse events after 30 days and 6 months of follow-up in patients with acute chest pain was assessed by Brennan and coworkers.[5] MPO was higher in patients with established AMI than in those without established AMI at presentation. Patients initially negative for troponin T but subsequently positive for troponin T had higher MPO concentrations than patients with no increase in troponin T. Patients with high MPO (upper vs lower quartile) were more likely to have a major cardiac event at 30 days [OR 4.7 (95% CI, 2.8 –7.7)] and 6 months [OR 4.7 (95% CI, 2.9 –7.7)] of follow-up. Khan et al. studied the incidence of nonfatal myocardial infarction and death in 384 patients with ST-segment myocardial infarction and observed that those with MPO values above the median had an almost 7-fold (HR 6.91 [95% CI, 1.79–26.73]) increased risk of adverse outcomes.[10] In a study by Cavusoglu et al., MPO was found to be independently associated with MI [OR 1.60 (95% CI, 1.09–2.36)] in 193 men with ACS during 2 years of follow-up.[7] Another cross-sectional study showed

that MPO had a higher diagnostic sensitivity and specificity in identifying patients with AMI than the total white blood cell count and 3-chlorotyrosine.[24]

All in all, these studies indicate that measurement of MPO in patients presenting with acute chest pain provides clinically relevant information. It should be noted, however, that between-study differences in cutoff values for both MPO and the cardiac troponins might have affected the diagnostic and prognostic value of MPO compared to troponins.

MPO as a causal factor in the pathogenesis of atherosclerosis

MPO is important in host defense against pathogens. On the other hand, as discussed above, a considerable number of epidemiological and clinical studies have demonstrated an association between increased concentrations of MPO and CVD, independent of classical risk factors. The source of MPO in plasma is activated leukocytes. Release of MPO and subsequent formation of reactive species (MPO-derived reactive species)[3] may be triggered by several mechanisms. First, it is known that inflammation induces recruitment and activation of white blood cells. Second, minimally modified LDL particles in the intima may trigger the influx of monocytes that mature into resident macrophages, some of which express MPO. Third, neutrophils in the blood stream are attracted and bound to sites of damaged endothelium. MPO released by these adherent leukocytes is initially bound to the vascular endothelium and subsequently transcytosed to the subendothelial matrix.[25] Therefore, both local release by resident macrophages and transcytosis of intraluminally produced MPO are sources of MPO in the vascular wall.

The microenvironment of the subendothelial space is especially conducive to MPO activity. Mitochondrial respiration, NAD(P)H oxidases, xanthine oxidase, and uncoupled nitric oxide synthase (NOS) are major sources of the highly reactive superoxide radical, which is actively converted into hydrogen peroxide by superoxide dismutase. Although less reactive than the superoxide radical, hydrogen peroxide is the cosubstrate for all MPO-catalyzed reactions. MPO amplifies the oxidative potential of hydrogen peroxide by producing a variety of reactive oxidants, including chlorinating and nitrating species. In the following sections some targets of MPO-derived reactive substances are briefly described, but it should be noted that many more cellular macromolecules and processes are adversely affected by vascular

MPO activity. Key features of the adverse role of MPO in the vascular wall are depicted in Figure 1.

LDL oxidation by MPO

The oxidative modification of LDL is an early event in atherosclerosis, and oxidized LDL contributes to atherogenesis by promoting cholesterol deposition and transformation of macrophages into foam cells.[2]

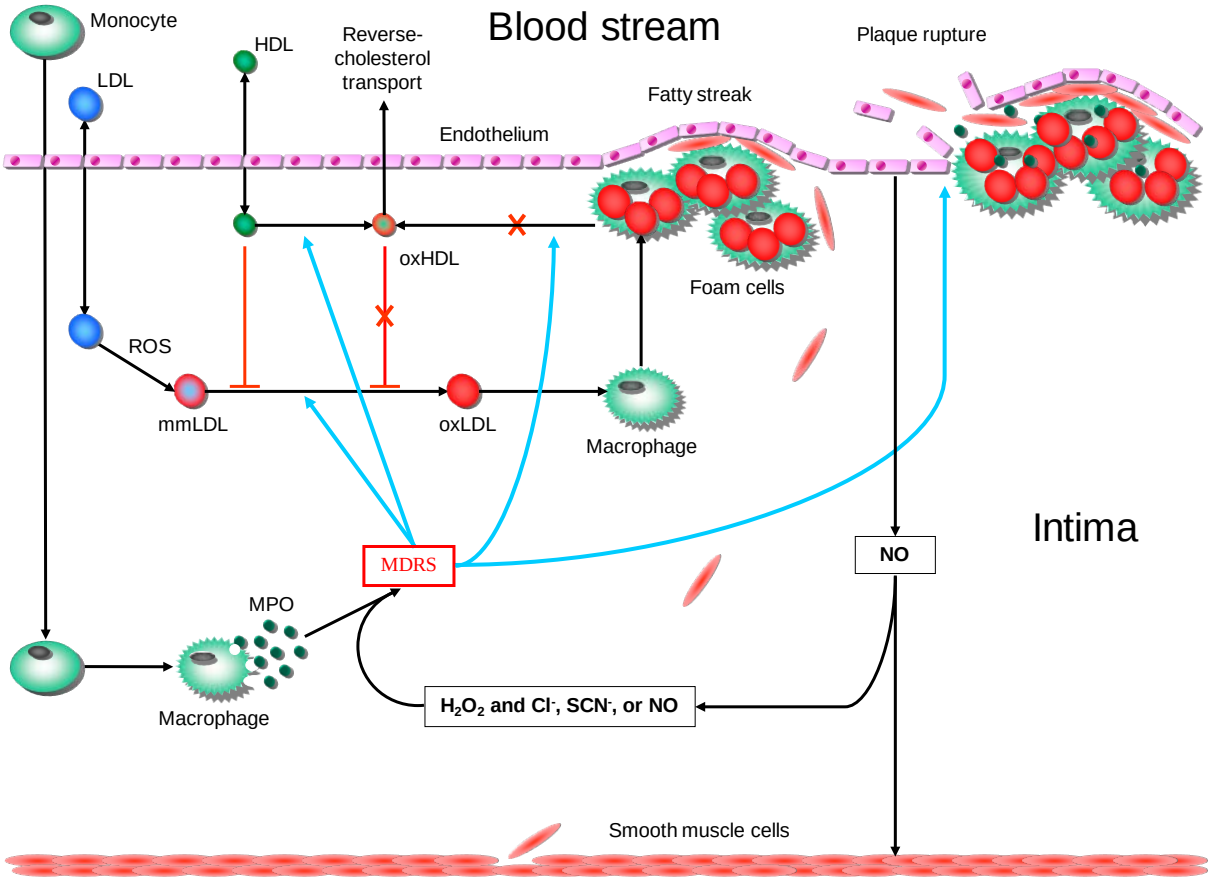

Figure 1. Adverse effects of MPO in the vasculature.
LDL particles penetrating the arterial intima may be minimally modified (mmLDL) by reactive oxygen species (ROS). Subsequently, mmLDL induces monocytes to migrate into the vascular wall, where they differentiate into macrophages. Oxidized LDL (oxLDL) is recognized by scavenger receptors of macrophages, and excess uptake leads to foam cell formation. MPO is released by macrophages in a state of inflammation and catalyzes the formation of myeloperoxidase-derived reactive species (MDRS) using chloride, thiocyanate, or NO as substrate and hydrogen peroxide as cosubstrate. Scavenging of NO may result in impaired vasodilation. Moreover, MDRS may promote atherosclerosis in different ways as indicated by the blue arrows. MDRS may oxidize LDL particles more extensively, forming oxidized LDL (oxLDL), and render HDL dysfunctional by promoting formation of oxidized HDL (oxHDL), thereby impairing HDL's protective effect on LDL particles and inhibiting reverse-cholesterol transport. MDRS may also destabilize plaques by weakening the fibrous cap.

Retention of LDL in the subendothelial space makes LDL a major target for oxidation by prooxidants produced by arterial wall cells. Sources of oxidants include NAD(P)H oxidases, xanthine oxidase, lipoxygenases, mitochondrial respiration, uncoupled NOS, and MPO. MPO is a highly cationic protein and can bind to endothelial cells, leukocytes, and LDL. The association of MPO with LDL may enhance oxidation of this lipoprotein.[26] MPO generates a number of reactive species, including hypochlorous acid, chloramines, tyrosyl radicals, and nitrogen dioxide, that oxidize the protein, lipid, and antioxidant constituents of LDL.[27] Many of the primary oxidation products are unstable and serve as reactive intermediates that promote further oxidative modifications of LDL and may also lead to cross-linking and aggregation. However, a limited number of stable oxidation products have been identified that may serve as biomarkers of MPO catalyzed oxidation. The modified tyrosine residues 3-nitrotyrosine and 3-chlorotyrosine are among the best characterized of these stable oxidation products.[28] Although MPO-generated reactive nitrogen species are involved in the conversion of tyrosine into 3-nitrotyrosine, other mechanisms may contribute to the formation of 3-nitrotyrosine as well. Notably, peroxynitrite, the reaction product of nitric oxide and superoxide, is also involved in the generation of 3-nitrotyrosine. In contrast to 3-nitrotyrosine, 3-chlorotyrosine is uniquely produced by MPO, and may therefore serve as a unique molecular fingerprint for MPO-catalyzed oxidation. Hazen and Heinecke reported that 3-chlorotyrosine was 6-fold higher in human advanced atherosclerotic lesions compared with normal aortic tissue. Moreover, the concentration of 3-chlorotyrosine in LDL isolated from atherosclerotic intima was 30-fold higher than in circulating LDL.[29] Mocatta et al.[12] measured chlorotyrosine in total plasma protein, however, and found no differences between groups that varied by 5-fold in mean MPO con-centration or by 20-fold in protein carbonyls. From these results they concluded that any hypochlorous acid generated by MPO in plasma is insufficient to be the source of protein carbonyls. Although 3-chlorotyrosine constitutes but a minor fraction of oxidation products in LDL, it carries a very specific MPO signature. Recently it has been shown that thiocyanate, which is abundantly present in plasma and is increased in smokers, is oxidized by MPO to cyanate that may, through carbamylation of proteins, alter their structure and function.[30] This study clearly showed that MPO-catalyzed carbamylation might enhance the proatherogenic properties of LDL.

Taken together, these data strongly suggest that MPO is implicated in oxidative modification of LDL in the vascular intima, resulting in conversion of LDL into an atherogenic form.

Impairment of HDL function by MPO

In addition to playing a central role in cholesterol efflux and reverse-cholesterol transport, HDL also possesses anti-inflammatory and antioxidative properties.[31] Although the exact mechanisms are not fully understood, some of the apolipoproteins and enzymes associated with HDL particles have antioxidative capacities. In this respect the protective role of HDL in MPO-mediated LDL oxidation is important. Mechanisms by which HDL can prevent or delay oxidation in the vessel wall include binding of transition metal ions and removal of oxidized (phospho)lipids and shortchain aldehydes from cells and LDL. After uptake by HDL, these oxidation products are either hydrolyzed by HDL-associated enzymes, such as platelet-activating factor, acetylhydrolase, and paraoxonase; or remain associated with HDL and are eventually eliminated from the circulation after hepatic uptake of HDL. The anti-inflammatory activity of HDL is probably closely linked to its antioxidative activity, because many oxidized lipids possess potent pro-inflammatory properties and can trigger arterial inflammation. HDL particles decrease expression of adhesion molecules on endothelial cells and inhibit adhesion of monocytes to these cells and entry of inflammatory cells into the intima.

In metabolic diseases associated with accelerated atherosclerosis, HDL particles may become functionally defective.[31] Dysfunctional HDL particles lack atheroprotective properties and promote pro-inflammatory effects. Over the past few years it has become clear that MPO is involved in rendering HDL dysfunctional.[32-34] Apolipoprotein A-I (apoA-I) is a preferred target for MPO-catalyzed oxidation, as evidenced by an approximately 100-fold enrichment of both 3-nitrotyrosine and 3-chlorotyrosine in apoA-I recovered from circulatory HDL compared to other proteins in the circulation.[35] Moreover, enrichment of these oxidative modifications in apoA-I isolated from human atherosclerotic plaques was even higher. A higher 3-nitrotyrosine and 3-chlorotyrosine content of HDL was significantly associated with diminished cholesterol efflux capacity.[35,36] A strong association between apoA-I modification and the prevalence of CVD has been observed. Individuals with an

apoA-I 3-chlorotyrosine content in the highest tertile were 16-fold more likely to have CVD compared to those with apoA-I 3-chlorotyrosine in the lowest tertile.[35] Selective targeting of apoA-I is explained by the fact that apoA-I contains a specific binding site for MPO. [35,36] Importantly, HDL-bound MPO retains its enzymatic activity, and binding to HDL may protect MPO from cellular uptake and degradation. Interestingly, MPO-dependent modification of HDL markedly increases the binding affinity of HDL for MPO, which has been proposed to lead to a vicious cycle of MPO dependent modifications at sites of chronic inflammation.[37] It should be noted that nitrotyrosine and chlorotyrosine in particular serve as molecular fingerprints for involvement of MPO in oxidative modification of HDL, but most likely other MPO-catalyzed modifications are quantitatively more important in generating dysfunctional HDL.

MPO reduces the bioavailability of nitric oxide

NO produced by endothelial NOS is a powerful vasodilator and as such plays a critical role in the regulation of vascular tone. Additionally, NO suppresses binding of circulating cells to the endothelium and inhibits proliferation of smooth muscle cells in the vascular wall (Figure 1). Taken together, these findings indicate that NO is a critical element in vascular homeostasis, and consequently insufficient production and/or increased scavenging of NO may impair vascular function and accelerate atherosclerosis. There are strong indications that MPO, by several mechanisms, may reduce the bioavailability of NO. First, NO serves as a substrate for peroxidases and MPO may thus serve as a catalytic sink for NO.[38] Second, scavenging of NO by MPO derived reactive substances may further reduce the bioavailability of NO. Third, hypochlorous acid can react with nitrogen atoms of the NOS substrate arginine to produce chlorinated arginine species that are inhibitors of all isoforms of NOS and have been shown to impair endothelium-dependent relaxation of rat aortic rings.[39] Finally, it has been demonstrated that hypochlorous acid is a potent inducer of uncoupling of endothelial NOS, thereby turning NOS into a superoxide-producing enzyme.[40] Although the relative impact of these mechanisms is currently unknown, it is clear that MPO, by catalytic as well as noncatalytic processes, depletes NO in the vascular wall. In agreement with this notion was the observation, in human study participants, of a strong inverse association between MPO serum concentrations and brachial artery flow-mediated dilation, which remained significant after adjustment for

classic CVD risk factors and CRP.[41] In a study by Baldus et al., release of vascular MPO from the subendothelial space by intravenous administration of heparin resulted in an improvement of endothelium-dependent vascular function, reflected by increases of brachial flow-mediated dilation and acetylcholine-induced forearm blood flow.[42]

MPO and plaque vulnerability

Plaque destabilization and rupture are thought to be essential processes in inducing acute cardiovascular events. For example Rossi et al. found a higher incidence of unstable plaques in patients with AMI.[20] MPO may play a role in plaque destabilization by activating metalloproteinases, thereby weakening the fibrous cap. This notion is supported by several studies that showed a positive association of MPO and CVD mortality in patients with acute cardiovascular events, whereas 2 studies that included patients with stable CAD failed to show a predictive value of MPO for CVD.[17,19] Additional evidence for a role of MPO in plaque destabilization is obtained from pathophysiologic studies.Plaque injury activates neutrophils, which may lead to MPO release.[43] Malle et al. observed colocalization of MPO and hypochlorite-modified proteins in human atherosclerotic lesions.[44] Sugiyama et al. reported that, in contrast to macrophages in fatty streaks that contain little or no MPO, macrophages in eroded or ruptured plaques are rich in MPO.[45] Consistent with this observation, these investigators found a higher concentration of proteins modified by hypochlorous acid in eroded and ruptured plaques compared to stable plaques. Taken together, existing data provide strong evidence that MPO may be involved in turning late-stage atherosclerosis into acute cardiovascular events.

Laboratory analyses of MPO: methodology and pitfalls

Differences in study populations or analytical procedures may influence the estimated predictive value of MPO in CVD risk stratification. Most results from the above-mentioned studies are based on measurement of MPO mass. Although a high correlation between MPO mass and MPO activity (r = 0.95) has been reported,[15] it cannot be completely ruled out that measurement of MPO activity instead of concentration would have influenced the results. As a matter of fact, the preferred assay procedure (mass or activity) is currently not known. It should be noted,

however, that ex vivo measurement of MPO activity does not necessarily reflect the in vivo activity of the enzyme. Enzymatic activity of MPO strongly depends on the local concentration of the cosubstrate hydrogen peroxide, which is relatively high in the microenvironment of the subendothelial space, especially in individuals with risk factors associated with increased oxidative stress, such as smoking, hypertension, and type 2 diabetes. Laboratory measurement of MPO activity is usually performed in the presence of saturating amounts of substrate and hydrogen peroxide and therefore represents maximal enzyme activity that can most likely be regarded as a proxy for enzyme mass.

The type of analytical specimen also differs between studies; whole blood, leukocytes, and plasma have all been used. Additionally, measurement of MPO in plasma has been performed after an intravenous bolus injection of heparin, which mobilizes MPO from vascular compartments and therefore yields higher values compared to baseline plasma concentrations, especially in patients with CAD.[42] Heparin may induce MPO release in white blood cells,[46] and neutrophils are activated during coagulation of blood.[47] The effect of collection tubes on MPO concentrations has recently been studied in patients presenting with symptoms of ACS.[48] MPO concentrations were higher in serum and samples collected in heparin tubes than in samples collected in EDTA or citrate tubes. Moreover, samples collected into EDTA-tubes stored on ice or at room temperature were the most stable. Plasma MPO concentrations remained relatively stable in heparin samples stored on ice, but storage at room temperature for 2 hours led to a 4-fold increase in MPO concentrations. From these results, Shih and coworkers concluded that EDTA should be the preferred anticoagulant for samples collected for MPO determination.

An additional issue that hampers comparison of reported MPO concentrations is the fact that some authors report MPO concentrations on a molar or weight basis, whereas others normalize to total protein by reporting MPO/protein ratios.

Clearly, reported measures of MPO are not directly comparable and are dependent on the type of specimen used. In fact, quite a few reports do not include information on the anticoagulant used to obtain plasma. Thus standardization of method protocols and blood collection tube type is urgently required.

Concluding remarks

Epidemiological studies in a wide range of patient populations clearly indicate that MPO, in addition to traditional markers, is an important CVD risk marker, especially in patients with unstable CAD. The causative role of MPO in initiating CVD and acute cardiovascular events is supported by in vitro experiments and pathophysiological observations, indicating that MPO is involved in all stages of atherogenesis from endothelial dysfunction to plaque rupture. Before MPO can be used routinely in clinical practice for CVD risk stratification, however, a better understanding of and recommendations for preanalytical and analytical procedures are important.

References

1. Ross R. Atherosclerosis--an inflammatory disease. *N Engl J Med*. 1999;340:115-126.
2. Stocker R, Keaney JF, Jr. Role of oxidative modifications in atherosclerosis. *Physiol Rev*. 2004;84:1381-1478.
3. Klebanoff SJ. Myeloperoxidase: friend and foe. *J Leukoc Biol*. 2005;77:598-625.
4. Baldus S, Heeschen C, Meinertz T, Zeiher AM, Eiserich JP, Munzel T, Simoons ML, Hamm CW. Myeloperoxidase serum levels predict risk in patients with acute coronary syndromes. *Circulation*. 2003;108:1440-1445.
5. Brennan ML, Penn MS, Van Lente F, Nambi V, Shishehbor MH, Aviles RJ, Goormastic M, Pepoy ML, McErlean ES, Topol EJ, Nissen SE, Hazen SL. Prognostic value of myeloperoxidase in patients with chest pain. *N Engl J Med*. 2003;349:1595-1604.
6. Brevetti G, Schiano V, Laurenzano E, Giugliano G, Petretta M, Scopacasa F, Chiariello M. Myeloperoxidase, but not C-reactive protein, predicts cardiovascular risk in peripheral arterial disease. *Eur Heart J*. 2008;29:224-230.
7. Cavusoglu E, Ruwende C, Eng C, Chopra V, Yanamadala S, Clark LT, Pinsky DJ, Marmur JD. Usefulness of baseline plasma myeloperoxidase levels as an independent predictor of myocardial infarction at two years in patients presenting with acute coronary syndrome. *Am J Cardiol*. 2007;99:1364-1368.
8. Dominguez-Rodriguez A, Samimi-Fard S, Abreu-Gonzalez P, Garcia-Gonzalez MJ, Kaski JC. Prognostic value of admission myeloperoxidase levels in patients with ST-segment elevation myocardial infarction and cardiogenic shock. *Am J Cardiol*. 2008;101:1537-1540.
9. Exner M, Minar E, Mlekusch W, Sabeti S, Amighi J, Lalouschek W, Maurer G, Bieglmayer C, Kieweg H, Wagner O, Schillinger M. Myeloperoxidase predicts progression of carotid stenosis in states of low high-density lipoprotein cholesterol. *J Am Coll Cardiol*. 2006;47:2212-2218.
10. Khan SQ, Kelly D, Quinn P, Davies JE, Ng LL. Myeloperoxidase aids prognostication together with N-terminal pro-B-type natriuretic peptide in high-risk patients with acute ST elevation myocardial infarction. *Heart*. 2007;93:826-831.
11. Meuwese MC, Stroes ES, Hazen SL, Van Miert JN, Kuivenhoven JA, Schaub RG, Wareham NJ, Luben R, Kastelein JJ, Khaw KT, Boekholdt SM. Serum myeloperoxidase levels are associated with the future risk of coronary artery disease in apparently healthy individuals: the EPIC-Norfolk Prospective Population Study. *J Am Coll Cardiol*. 2007;50:159-165.
12. Mocatta TJ, Pilbrow AP, Cameron VA, Senthilmohan R, Frampton CM, Richards AM, Winterbourn CC. Plasma concentrations of myeloperoxidase predict mortality after myocardial infarction. *J Am Coll Cardiol*. 2007;49:1993-2000.
13. Morrow DA, Sabatine MS, Brennan ML, De Lemos JA, Murphy SA, Ruff CT, Rifai N, Cannon CP, Hazen SL. Concurrent evaluation of novel cardiac biomarkers in acute coronary syndrome: myeloperoxidase and soluble CD40 ligand and the risk of recurrent ischaemic events in TACTICS-TIMI 18. *Eur Heart J*. 2008;29:1096-1102.

14. Ndrepepa G, Braun S, Mehilli J, Von Beckerath N, Schomig A, Kastrati A. Myeloperoxidase level in patients with stable coronary artery disease and acute coronary syndromes. *Eur J Clin Invest*. 2008;38:90-96.
15. Zhang R, Brennan ML, Fu X, Aviles RJ, Pearce GL, Penn MS, Topol EJ, Sprecher DL, Hazen SL. Association between myeloperoxidase levels and risk of coronary artery disease. *JAMA*. 2001;286:2136-2142.
16. Duzguncinar O, Yavuz B, Hazirolan T, Deniz A, Tokgozoglu SL, Akata D, Demirpence E. Plasma myeloperoxidase is related to the severity of coronary artery disease. *Acta Cardiol*. 2008;63:147-152.
17. Stefanescu A, Braun S, Ndrepepa G, Koppara T, Pavaci H, Mehilli J, Schomig A, Kastrati A. Prognostic value of plasma myeloperoxidase concentration in patients with stable coronary artery disease. *Am Heart J*. 2008;155:356-360.
18. El Bejjani D, Hazen SL, Mackay W, Glass NE, Hulgan T, Tungsiripat M, McComsey GA. Higher plasma myeloperoxidase levels are not associated with an increased risk for cardiovascular events in HIV-infected adults. *HIV Clin Trials*. 2008;9:207-211.
19. Kubala L, Lu G, Baldus S, Berglund L, Eiserich JP. Plasma levels of myeloperoxidase are not elevated in patients with stable coronary artery disease. *Clin Chim Acta*. 2008;394:59-62.
20. Rossi A, Franceschini L, Fusaro M, Cicoira M, Eleas AA, Golia G, Bonapace S, Santini F, Sangiorgi G, Zardini P, Vassanelli C. Carotid atherosclerotic plaque instability in patients with acute myocardial infarction. *Int J Cardiol*. 2006;111:263-266.
21. Apple FS, Pearce LA, Chung A, Ler R, Murakami MM. Multiple biomarker use for detection of adverse events in patients presenting with symptoms suggestive of acute coronary syndrome. *Clin Chem*. 2007;53:874-881.
22. Esporcatte R, Rey HC, Rangel FO, Rocha RM, Mendonca Filho HT, Dohmann HF, Albanesi Filho FM. Predictive value of myeloperoxidase to identify high risk patients admitted to the hospital with acute chest pain. *Arq Bras Cardiol*. 2007;89:377-384.
23. Apple FS, Smith SW, Pearce LA, Murakami MM. Assessment of the multiple-biomarker approach for diagnosis of myocardial infarction in patients presenting with symptoms suggestive of acute coronary syndrome. *Clin Chem*. 2009;55:93-100.
24. Cheng ML, Chen CM, Gu PW, Ho HY, Chiu DT. Elevated levels of myeloperoxidase, white blood cell count and 3-chlorotyrosine in Taiwanese patients with acute myocardial infarction. *Clin Biochem*. 2008;41:554-560.
25. Baldus S, Eiserich JP, Mani A, Castro L, Figueroa M, Chumley P, Ma W, Tousson A, White CR, Bullard DC, Brennan ML, Lusis AJ, Moore KP, Freeman BA. Endothelial transcytosis of myeloperoxidase confers specificity to vascular ECM proteins as targets of tyrosine nitration. *J Clin Invest*. 2001;108:1759-1770.
26. Carr AC, Myzak MC, Stocker R, McCall MR, Frei B. Myeloperoxidase binds to low-density lipoprotein: potential implications for atherosclerosis. *FEBS Lett*. 2000;487:176-180.

27. Carr AC, McCall MR, Frei B. Oxidation of LDL by myeloperoxidase and reactive nitrogen species: reaction pathways and antioxidant protection. *Arterioscler Thromb Vasc Biol*. 2000;20:1716-1723.
28. Podrez EA, Abu-Soud HM, Hazen SL. Myeloperoxidase-generated oxidants and atherosclerosis. *Free Radic Biol Med*. 2000;28:1717-1725.
29. Hazen SL, Heinecke JW. 3-Chlorotyrosine, a specific marker of myeloperoxidase-catalyzed oxidation, is markedly elevated in low density lipoprotein isolated from human atherosclerotic intima. *J Clin Invest*. 1997;99:2075-2081.
30. Wang Z, Nicholls SJ, Rodriguez ER, Kummu O, Horkko S, Barnard J, Reynolds WF, Topol EJ, DiDonato JA, Hazen SL. Protein carbamylation links inflammation, smoking, uremia and atherogenesis. *Nat Med*. 2007;13:1176-1184.
31. Kontush A, Chapman MJ. Functionally defective high-density lipoprotein: a new therapeutic target at the crossroads of dyslipidemia, inflammation, and atherosclerosis. *Pharmacol Rev*. 2006;58:342-374.
32. Panzenboeck U, Raitmayer S, Reicher H, Lindner H, Glatter O, Malle E, Sattler W. Effects of reagent and enzymatically generated hypochlorite on physicochemical and metabolic properties of high density lipoproteins. *J Biol Chem*. 1997;272:29711-29720.
33. Nicholls SJ, Zheng L, Hazen SL. Formation of dysfunctional high-density lipoprotein by myeloperoxidase. *Trends Cardiovasc Med*. 2005;15:212-219.
34. Shao B, Oda MN, Oram JF, Heinecke JW. Myeloperoxidase: an inflammatory enzyme for generating dysfunctional high density lipoprotein. *Curr Opin Cardiol*. 2006;21:322-328.
35. Zheng L, Nukuna B, Brennan ML, Sun M, Goormastic M, Settle M, Schmitt D, Fu X, Thomson L, Fox PL, Ischiropoulos H, Smith JD, Kinter M, Hazen SL. Apolipoprotein A-I is a selective target for myeloperoxidase-catalyzed oxidation and functional impairment in subjects with cardiovascular disease. *J Clin Invest*. 2004;114:529-541.
36. Bergt C, Pennathur S, Fu X, Byun J, O'Brien K, McDonald TO, Singh P, Anantharamaiah GM, Chait A, Brunzell J, Geary RL, Oram JF, Heinecke JW. The myeloperoxidase product hypochlorous acid oxidizes HDL in the human artery wall and impairs ABCA1-dependent cholesterol transport. *Proc Natl Acad Sci U S A*. 2004;101:13032-13037.
37. Marsche G, Furtmuller PG, Obinger C, Sattler W, Malle E. Hypochlorite-modified high-density lipoprotein acts as a sink for myeloperoxidase in vitro. *Cardiovasc Res*. 2008;79:187-194.
38. Abu-Soud HM, Hazen SL. Nitric oxide is a physiological substrate for mammalian peroxidases. *J Biol Chem*. 2000;275:37524-37532.
39. Yang J, Ji R, Cheng Y, Sun JZ, Jennings LK, Zhang C. L-arginine chlorination results in the formation of a nonselective nitric-oxide synthase inhibitor. *J Pharmacol Exp Ther*. 2006;318:1044-1049.
40. Xu J, Xie Z, Reece R, Pimental D, Zou MH. Uncoupling of endothelial nitric oxidase synthase by hypochlorous acid: role of NAD(P)H oxidase-derived superoxide and peroxynitrite. *Arterioscler Thromb Vasc Biol*. 2006;26:2688-2695.

41. Vita JA, Brennan ML, Gokce N, Mann SA, Goormastic M, Shishehbor MH, Penn MS, Keaney JF, Jr., Hazen SL. Serum myeloperoxidase levels independently predict endothelial dysfunction in humans. *Circulation*. 2004;110:1134-1139.
42. Baldus S, Rudolph V, Roiss M, Ito WD, Rudolph TK, Eiserich JP, Sydow K, Lau D, Szocs K, Klinke A, Kubala L, Berglund L, Schrepfer S, Deuse T, Haddad M, Risius T, Klemm H, Reichenspurner HC, Meinertz T, Heitzer T. Heparins increase endothelial nitric oxide bioavailability by liberating vessel-immobilized myeloperoxidase. *Circulation*. 2006;113:1871-1878.
43. Rudolph V, Steven D, Gehling UM, Goldmann B, Rudolph TK, Friedrichs K, Meinertz T, Heitzer T, Baldus S. Coronary plaque injury triggers neutrophil activation in patients with coronary artery disease. *Free Radic Biol Med*. 2007;42:460-465.
44. Malle E, Waeg G, Schreiber R, Grone EF, Sattler W, Grone HJ. Immunohistochemical evidence for the myeloperoxidase/H2O2/halide system in human atherosclerotic lesions: colocalization of myeloperoxidase and hypochlorite-modified proteins. *Eur J Biochem*. 2000;267:4495-4503.
45. Sugiyama S, Okada Y, Sukhova GK, Virmani R, Heinecke JW, Libby P. Macrophage myeloperoxidase regulation by granulocyte macrophage colony-stimulating factor in human atherosclerosis and implications in acute coronary syndromes. *Am J Pathol*. 2001;158:879-891.
46. Leculier C, Couprie N, Adeleine P, Leitienne P, Francina A, Richard M. The effects of high molecular weight- and low molecular weight-heparins on superoxide ion production and degranulation by human polymorphonuclear leukocytes. *Thromb Res*. 1993;69:519-531.
47. De Gaetano G, Cerletti C, Evangelista V. Recent advances in platelet-polymorphonuclear leukocyte interaction. *Haemostasis*. 1999;29:41-49.
48. Shih J, Datwyler SA, Hsu SC, Matias MS, Pacenti DP, Lueders C, Mueller C, Danne O, Mockel M. Effect of collection tube type and preanalytical handling on myeloperoxidase concentrations. *Clin Chem*. 2008;54:1076-1079.

Chapter 4

Myeloperoxidase concentrations in EDTA-plasma of healthy subjects are discordant with concentrations in heparin-plasma and serum

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Abstract

Objectives: To examine the effect of blood anticoagulation type on the myeloperoxidase (MPO) concentration.

Design and methods: MPO was measured in EDTA-plasma and matched heparin-plasma and serum samples collected from healthy volunteers.

Results: MPO concentrations in heparin-plasma and serum were higher than in EDTA-plasma (both $P<0.001$). MPO in EDTA-plasma was not significantly correlated with MPO in either heparin-plasma or serum.

Conclusions: For MPO measurements, EDTA-plasma is the preferred specimen as it appears unaffected by ex vivo release of MPO from leukocytes.

Introduction

Myeloperoxidase (MPO) is released upon activation of leukocytes and is important in host defense against pathogens.[1] Paradoxically, epidemiological and clinical studies have demonstrated a positive association between MPO and cardiovascular disease, independent of classical risk factors.[2,3] The link between MPO and cardiovascular disease has resulted in an upsurge in research in this field. Measurement of MPO mass is rather easy, but there is no consensus on which type of anticoagulation is preferable for its measurement. Commercial kits are available from numerous suppliers. Some manufacturers recommend measurement in heparin-plasma (e.g. Northwest, Prognostix, and Assay Designs/Stressgen) or serum/EDTA-plasma (e.g. Mercodia, Immunodiagnostik), while other companies do not advise a specific specimen (e.g. Abbott Diagnostics). The effect of collection tubes on MPO concentrations has recently been studied in patients presenting with symptoms of acute coronary syndrome.[4] Although absolute MPO values differed considerably between tube types, correlation coefficients appeared to be quite reasonable in this study (r=0.89 for EDTA-plasma versus heparin-plasma, and r=0.82 for EDTA-plasma versus serum). Since blood collected from patients presenting with symptoms of acute coronary syndromes was used, the MPO concentrations encompassed a very wide range (~100-fold) and had a right-skewed distribution. As a consequence, the reported correlation coefficients were probably inflated by inclusion of extreme MPO values, and a weaker association in the normal range of MPO concentrations may be expected. Therefore, the aim of our study was to compare MPO concentrations measured in matched serum and plasma samples collected from apparently healthy volunteers.

Materials and methods

The study group, recruited among laboratory personnel and medical students, consisted of 27 men and 62 women, with a median (interquartile range) age of 25 (23–28) years. All participants gave informed consent to take part in the study.

Non-fasting blood samples were collected into plastic tubes containing K_2-EDTA or lithium heparin as anticoagulant and in glass tubes without an anticoagulant and clot activator (BD Vacutainer® blood collection tubes 367864, 368886 and 367615, respectively). None of the tubes contained a separating gel. Blood samples were

kept at room temperature for ~1 h. Then the tubes were centrifuged at 1500 g for 10 min. Plasma and serum were carefully pipetted to minimize transfer of white blood cells. Aliquots were stored immediately afterwards at −80 °C until analysis. Two groups were used for MPO comparison in EDTA-plasma versus heparin-plasma (n=40) and EDTA-plasma versus serum (n=41). In addition, from a relatively small group of participants (n=8) we collected EDTA-, heparin-plasma as well as serum.

MPO was measured in duplicate by a sandwich ELISA (Mercodia, Uppsala, Sweden). The intra-assay and inter-assay coefficients of variation were 3.9% and 5.0%, respectively for MPO measured in EDTA-plasma, and measurement in heparin-plasma and serum was performed with similar precision. In a previous study we have shown that the concentration of MPO differs only slightly between the fasting and postprandial state.[5]

Results and discussion

MPO concentrations measured in EDTA-plasma were compared with values obtained in matched heparin-plasma (n=48) and serum (n=49) samples.

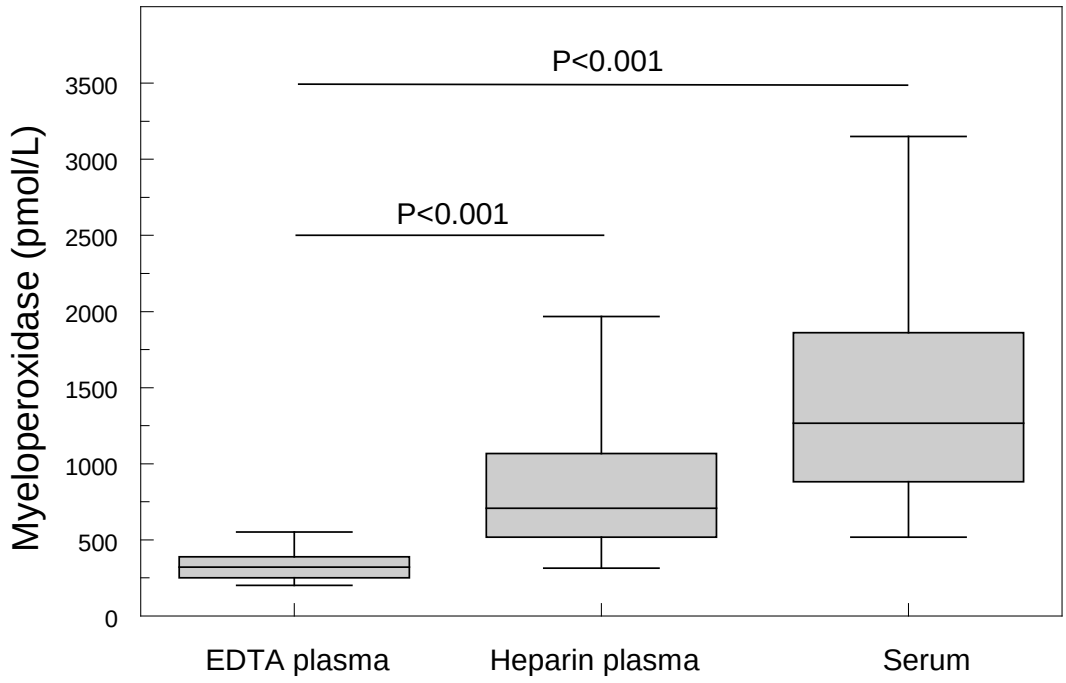

Figure 1. Box-whisker plot of myeloperoxidase concentrations measured in EDTA-plasma (n=89), heparin-plasma (n=48) and serum (n=49) of healthy volunteers.

The median (interquartile range) MPO concentration was 321 (250–389) pmol/L in EDTA-plasma, 708 (519–1067) pmol/L in heparin-plasma, and 1265 (882–1849) pmol/L in serum. As illustrated in Figure 1, MPO concentrations in heparin-plasma and serum were significantly higher than in EDTA-plasma (both $P<0.001$; Wilcoxon Signed Rank test). This observation is in agreement with a recently published paper about the effect of collection tubes on MPO in patients presenting with symptoms of acute coronary syndrome.[4]

The higher MPO concentrations in heparin-plasma and serum can be explained by in vitro release of MPO from activated leukocytes by heparin-stimulated degranulation[6] and during blood clotting.[7] As shown in Figure 2, MPO measured in EDTA-plasma did not significantly correlate with MPO concentrations in either heparin-plasma ($r_s=0.25$, $P=0.09$) or serum ($r_s=0.13$, $P=0.37$).

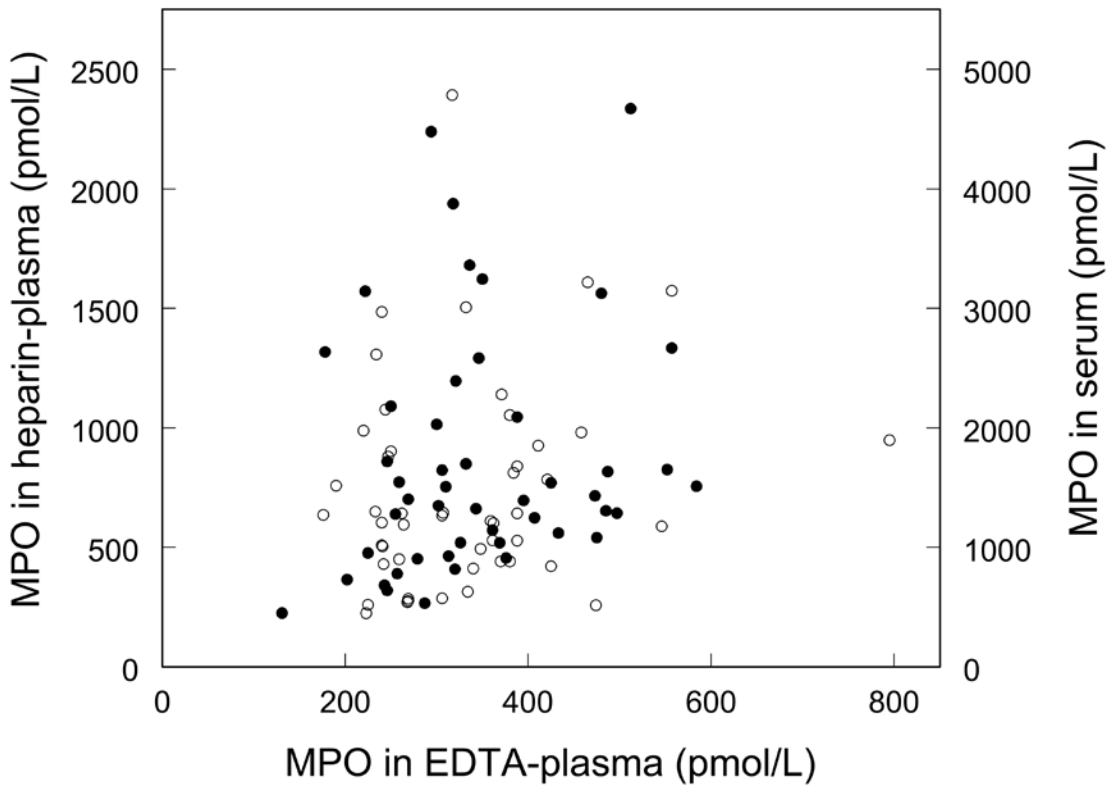

Figure 2. Scatter plots of myeloperoxidase (MPO) concentrations measured in EDTA-plasma versus concentrations measured in heparin-plasma (closed circles; $r_s=0.25$, $P=0.09$) and serum (open circles; $r_s=0.13$, $P=0.37$).

It has been shown that samples collected into EDTA-tubes stored on ice or at room temperature were the most stable.[8] Additionally, plasma MPO concentrations remained relatively stable in heparin samples stored on ice, but storage at room

temperature for 2 h led to a 4-fold increase in MPO concentrations. Of note, we have previously shown that MPO concentrations do not change over time in blood collected in EDTA-tubes and kept for 2 h at room temperature before processing, whereas MPO rose gradually in heparin-plasma as well as in blood drawn in tubes without an added anticoagulant.[5] From these results, it can be concluded that EDTA should be the preferred anticoagulant for blood samples collected for MPO determination.

In conclusion, MPO concentrations in the normal range measured in EDTA-plasma were discordant with concentrations measured in matched heparin-plasma or serum samples. EDTA-plasma is recommended for MPO measurement because values are not confounded by poorly controllable ex vivo release of MPO from leukocytes and may therefore most accurately reflect the concentration of MPO in the circulation.

Acknowledgments

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References

1. Winterbourn CC. Comparative reactivities of various biological compounds with myeloperoxidase-hydrogen peroxide-chloride, and similarity of the oxidant to hypochlorite. *Biochim Biophys Acta*. 1985;840:204-210.
2. Nicholls SJ, Hazen SL. Myeloperoxidase and cardiovascular disease. *Arterioscler Thromb Vasc Biol*. 2005;25:1102-1111.
3. Schindhelm RK, Van der Zwan LP, Teerlink T, Scheffer PG. Myeloperoxidase: a useful biomarker for cardiovascular disease risk stratification? *Clin Chem*. 2009;55:1462-1470.
4. Shih J, Datwyler SA, Hsu SC, Matias MS, Pacenti DP, Lueders C, Mueller C, Danne O, Mockel M. Effect of collection tube type and preanalytical handling on myeloperoxidase concentrations. *Clin Chem*. 2008;54:1076-1079.
5. Schindhelm RK, Alssema M, Diamant M, Teerlink T, Dekker JM, Kok A, Kostense PJ, Nijpels G, Heine RJ, Scheffer PG. Comparison of two consecutive fat-rich and carbohydrate-rich meals on postprandial myeloperoxidase response in women with and without type 2 diabetes mellitus. *Metabolism*. 2008;57:262-267.
6. Leculier C, Couprie N, Adeleine P, Leitienne P, Francina A, Richard M. The effects of high molecular weight- and low molecular weight-heparins on superoxide ion production and degranulation by human polymorphonuclear leukocytes. *Thromb Res*. 1993;69:519-531.
7. Gerasimov IG, Ignatov DY. Blood coagulation initiates respiratory burst in neutrophils. *Bull Exp Biol Med*. 2005;140:77-79.
8. Chang PY, Wu TL, Hung CC, Tsao KC, Sun CF, Wu LL, Wu JT. Development of an ELISA for myeloperoxidase on microplate: normal reference values and effect of temperature on specimen preparation. *Clin Chim Acta*. 2006;373:158-163.

Chapter 5

Plasma myeloperoxidase is inversely associated with endothelium-dependent vasodilation in elderly subjects with abnormal glucose metabolism

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Abstract

Myeloperoxidase (MPO), a biomarker related to inflammation, oxidative stress, and nitric oxide scavenging, has been shown to impair endothelium-dependent vasodilation. Because elevated hydrogen peroxide concentrations in diabetic vessels may enhance MPO activity, we hypothesized that a stronger association of MPO with flow-mediated dilation (FMD) may be found in subjects with abnormal glucose metabolism. Myeloperoxidase concentrations were measured in EDTA-plasma samples from participants of a population-based cohort study, including 230 subjects with normal glucose metabolism and 386 with abnormal glucose metabolism. Vascular function was expressed as FMD and nitroglycerin-mediated dilation of the brachial artery. In subjects with abnormal glucose metabolism, MPO was negatively associated with FMD (−20.9 [95% confidence interval {CI}, −41.7 to −0.2] -µm change in FMD per SD increment of MPO). This association remained significant after adjustment for nitroglycerin-mediated dilation (−31.1 [95% CI, −50.0 to −12.3]) and was not attenuated after further adjustment for established risk factors. In subjects with normal glucose metabolism, MPO was not significantly associated with FMD (2.0 [95% CI, −16.0 to 20.0]). In conclusion, in subjects with abnormal glucose metabolism, plasma levels of MPO are inversely associated with endothelium-dependent vasodilation, possibly reflecting enhancement of MPO activity by vascular oxidative stress.

Introduction

Atherosclerosis is considered a disease involving inflammation, nitric oxide (NO) scavenging, and oxidative stress. Myeloperoxidase (MPO, EC 1.11.1.7) is a novel risk marker particularly useful to identify patients with acute cardiovascular disease (CVD).[1-5] Myeloperoxidase is released upon activation of polymorphonuclear cells and monocytes and is important in host defense against pathogens by producing hypochlorous acid and other highly reactive antimicrobial compounds like hypobromous acid, cyanate, chloramines, and tyrosyl and hydroxyl radicals.[6-9] These highly reactive compounds may, by promoting oxidation of low-density lipoprotein (LDL) and impairing high-density lipoprotein's (HDL's) anti-inflammatory properties and reverse-cholesterol transport functions, be considered proatherogenic.[6,9,10] Nitric oxide serves as a substrate for peroxidases, and MPO may as such serve as a catalytic sink for NO.[11] Scavenging of NO by MPO-derived oxidants may further reduce the bioavailability of this powerful vasodilator. In agreement with this notion, in patients undergoing coronary angiography, heparin was shown to liberate vessel-associated MPO, increase NO availability, and improve endothelium-dependent vascular function.[3] An inverse association between MPO serum concentrations and brachial artery flow-mediated dilation (FMD) has been observed in a hospital-based population of whom 51% had CVD.[12] However, to the best of our knowledge, the relationship between MPO and vascular function in the general population has never been assessed. Notably, the activity of MPO depends on the presence of the cosubstrate hydrogen peroxide. Production of reactive oxygen species, including superoxide and its dismutation product hydrogen peroxide, is increased in diabetic vessels and is important in the pathogenesis of diabetic vascular complications.[13,14] Consequently, MPO may amplify hyperglycemia-induced endothelial dysfunction. Therefore, the aim of our study was to assess in the general population if the plasma concentration of MPO is associated with endothelium-dependent vasodilation and, if so, whether this association is modified by glucose metabolism status.

Materials and methods

Subjects

The present study was conducted in the Hoorn Study follow-up examination[15] and the Hoorn Screening Study,[16] which are population-based studies in a white

population. From the 822 participants, we excluded subjects with missing data on primary variables of interest (n = 72) and subjects using lipid-lowering medication (n = 134) because these drugs may influence MPO and FMD values.[17] In total, 616 subjects (298 men and 318 women) remained, of whom 230 had normal glucose metabolism,159 had impaired glucose metabolism, and 227 had type 2 diabetes mellitus according to the WHO-99 criteria.[18] Abnormal glucose metabolism (n = 386) was defined as either impaired glucose metabolism or type 2 diabetes mellitus. Measurement of vascular function and withdrawal of blood samples were done after an overnight fast. The local ethics committee approved the study, and all participants gave their written informed consent.

Biochemical analysis

A sandwich enzyme-linked immunosorbent assay (ELISA) (Mercodia, Uppsala, Sweden) was used to determine MPO concentrations in EDTA-plasma, with intra- and interassay coefficients of variation of 3.3% and 5.0%, respectively.[19] Plasma C-reactive protein (CRP) concentrations were determined with a highly sensitive in-house sandwich ELISA.[20] Circulating plasma oxidized LDL (oxLDL) was determined by competitive ELISA (Mercodia). Oxidized LDL was expressed as the oxLDL to apolipoprotein (Apo) B-100 ratio to adjust for LDL particle number.[21] Glycated hemoglobin (HbA1C) was analyzed by ion-exchange high-performance liquid chromatography (reference range, 4.3%-6.1%) on a modular monitoring system (Bio-Rad, Veenendaal, the Netherlands). Glucose was measured enzymatically (Roche, Mannheim, Germany), and insulin was determined by a 2-site immunoradiometric assay (Medgenix Diagnostics, Fleurus, Belgium). Total cholesterol, high-density lipoprotein (HDL) cholesterol, and triglycerides were measured by standard enzymatic methods (Roche). Low-density lipoprotein cholesterol concentration was determined with a direct method by the N-geneous assay (GenZyme, Cambridge, MA). With this method, triglyceride concentrations up to 13.5 mmol/L do not interfere with measurement of LDL cholesterol. Apolipoprotein B-100 concentrations were determined nephelometrically using an Immage 800 immunochemistry system (Beckman Coulter, Fullerton, CA).

Vascular properties

Ultrasound examination of the right brachial artery was performed according to the guidelines of the International Brachial Artery Reactivity Task Force.[22] Baseline diameter, blood flow (peak systolic velocity), FMD, and nitroglycerin-mediated dilation (NMD) were determined by a single observer (RMAH) as previously described.[23] The intraobserver coefficients of variation were 4.3% for diameter, 14.7% for FMD, and 10.3% for NMD, respectively. Qualitatively satisfactory ultrasound examinations were obtained in 475 individuals (188 with normal glucose metabolism and 287 with abnormal glucose metabolism). Poor definition of the arterial wall due to obesity and inability to remain motionless due to musculoskeletal disorders were the main reasons for missing ultrasound data.[23]

Other measurements

Microalbuminuria was defined as urinary albumin to creatinine ratio of at least 2.0 mg/mmol. Prior CVD was defined as Minnesota Code 1.1 to 1.3, 4.1 to 4.3, 5.1 to 5.3, or 7.1 on the electrocardiogram; coronary bypass operation or angioplasty; an ankle-brachial blood pressure index less than 0.9 in either leg; peripheral arterial bypass; or amputation for atherosclerotic disease. The Framingham risk score was calculated.[24] Data on smoking status and on ascorbic acid, retinol, and tocopherol daily intake were obtained by questionnaire.[25]

Statistical analysis

Data are presented as means and standard deviations or, if skewed, median and interquartile range. Skewed variables, that is, insulin, triglycerides, CRP, and vitamin intake, were $\log_e$ transformed before trend analyses and linear regression analyses. Statistical significance for linear trend was calculated by analysis of variance or by linear-by-linear χ^2-tests. Differences in variables between normal glucose metabolism and abnormal glucose metabolism were tested by Mann-Whitney analyses. Associations of variables with MPO were tested by linear regression analyses adjusted for age and sex. Variables that were associated with MPO in these analyses (P <0.1) were used as independent variables in a multivariable linear regression model to establish which variables were independently associated with MPO. In regression models for FMD, we considered age, sex,

baseline diameter, and the increase in peak systolic velocity as standard correction variables. Regression models for NMD were adjusted for age, sex, baseline diameter, and glucose tolerance status. Effect modification by glucose metabolism status was investigated by including appropriate interaction terms in the models. Data were analyzed using SPSS software, version 15 (SPSS, Chicago, IL). A 2-tailed *P*-value <0.05 was considered to indicate statistical significance.

Results

Subjects characteristics

Subject characteristics by tertiles of MPO are shown in Table 1. Age, fasting glucose, HbA1C, insulin, free fatty acids, CRP, waist circumference, blood pressure, antihypertensive medication, prior CVD, and Framingham risk score increased across tertiles of MPO. In line with the increasing trends of variables related to glucose metabolism, the mean MPO concentration was higher in subjects with abnormal glucose metabolism than in subjects with normal glucose metabolism (60.2 ± 19.8 vs 57.3 ± 18.2 µg/L, *P* = 0.025). Higher tertiles of MPO were significantly associated with a lower daily intake of ascorbic acid (Table 1).

Table 1. Subject characteristics overall and by tertiles of plasma myeloperoxidase.

Variable	Unit	Overall	1st tertile	2nd tertile	3rd tertile	P_{trend}
Myeloperoxidase	µg/L		<50.6	50.6-62.7	>62.7	
N		616	206	206	204	
Age	years	69.0 (7.3)	68.4 (7.0)	68.5 (6.9)	70.2 (7.7)	0.014
Sex, male	%	48	54	42	49	0.32
Fasting glucose	mmol/L	6.4 (1.5)	6.3 (1.5)	6.3 (1.3)	6.7 (1.7)	<0.001
HbA_{1C}	%	6.1 (0.8)	5.9 (0.7)	6.1 (0.7)	6.2 (0.9)	<0.001
Insulin*	pmol/L	60 (42-88)	57 (43-78)	59(39-86)	67 (44-99)	0.003
Abnormal glucose metabolism	%	63	60	59	70	0.039
OxLDL/ApoB100	U/g	63.4 (9.0)	63.6 (9.1)	62.7 (9.0)	63.8 (9.1)	0.86
ApoB100	g/L	1.04 (0.23)	1.04 (0.25)	1.04 (0.22)	1.05 (0.23)	0.89
LDL-cholesterol	mmol/L	3.8 (0.9)	3.8 (0.9)	3.8 (0.8)	3.7 (0.9)	0.67
HDL-cholesterol	mmol/L	1.39 (0.41)	1.35 (0.40)	1.47 (0.42)	1.35 (0.39)	0.96
Triglycerides*	mmol/L	1.3 (1.0-1.8)	1.3 (1.0-1.7)	1.3 (1.0-1.7)	1.4 (1.0-2.0)	0.10
Free fatty acids	mmol/L	0.56 (0.24)	0.53 (0.21)	0.59 (0.24)	0.58 (0.26)	0.034
C-reactive protein*	mg/L	2.3 (1.1-4.8)	1.7 (0.9-2.9)	1.9 (1.1-4.3)	3.8 (2.0-7.3)	<0.001
Serum albumin	g/L	41.5 (2.9)	41.6 (2.6)	41.7 (3.1)	41.2 (3.0)	0.18
Waist circumference	cm	96 (13)	94 (11)	96 (12)	98 (14)	0.007
Systolic blood pressure	mmHg	142 (21)	140 (20)	141 (21)	146 (21)	0.002
Diastolic blood pressure	mmHg	83 (11)	82 (10)	83 (11)	84 (11)	0.029
Antihypertensives	%	33	29	29	43	0.003
Current smoking	%	16	11	21	17	0.12
Retinol intake*	mg/day	541 (393-869)	581 (392-904)	549 (412-878)	496 (366-766)	0.34
Ascorbic acid intake*	mg/day	97 (72-132)	107 (79-144)	96 (72-134)	89 (68-124)	0.001
Tocopherol intake*	mg/day	10.9 (8.3-14.6)	11.2 (8.6-14.9)	11.1 (8.5-14.4)	9.9 (7.6-14.5)	0.050
Micro-albuminuria	%	15	12	13	19	0.06
Prior cardiovascular disease	%	45	42	40	52	0.042
Framingham risk score		10.1 (3.5)	9.7 (3.3)	9.9 (3.4)	10.7 (3.8)	0.005

Values are displayed as means (SD), medians (interquartile range), or percentages. *P* for trend values were age- and sex adjusted. *Variables were $\log_e$-transformed prior to linear trend analysis.

Correlates of plasma MPO concentration

Age- and sex-adjusted analyses revealed positive significant associations between MPO and age, glucose, HbA1C, insulin, CRP, and blood pressure (Table 2). Myeloperoxidase was negatively associated with daily ascorbic acid intake. Myeloperoxidase was not significantly associated with sex, oxLDL/Apo B-100 ratio, Apo B-100, plasma lipids, serum albumin, waist circumference, smoking, retinol and tocopherol intake, microalbuminuria, and prior CVD. In a multivariable linear regression model, the only significant independent predictive variables were CRP (standardized β, 0.20; $P<0.001$) and ascorbic acid intake (standardized β, −0.11; $P = 0.007$). The proportion of variance (R^2) explained by this model was 0.086. Separate regression models for subjects with normal and abnormal glucose metabolism yielded essentially the same results as obtained for the entire cohort (data not shown).

Table 2. Age- and sex-adjusted associations with MPO.

Variable	Unit	Unstandardised Beta	*P*-value
Age	years	0.29 (0.08 to 0.50)	0.006
Sex	male vs. female	0.02 (-3.02 to 3.07)	0.99
Fasting glucose	mmol/L	1.30 (0.32 to 2.22)	0.010
HbA_{1C}	%	2.94 (0.97 to 4.91)	0.003
Insulin*	pmol/L	3.26 (0.45 to 6.07)	0.023
Abnormal glucose metabolism	yes vs. no	2.72 (-0.42 to 5.86)	0.09
OxLDL/ApoB100	U/g	-0.01 (-0.17 to 0.16)	0.96
ApoB100	g/L	-1.46 (-8.06 to 5.13)	0.66
LDL-cholesterol	mmol/L	-0.71 (-2.46 to 1.05)	0.43
HDL-cholesterol	mmol/L	-0.32 (-4.40 to 3.75)	0.88
Triglycerides*	mmol/L	0.63 (-2.72 to 3.98)	0.71
Free fatty acids	mmol/L	5.32 (-1.49 to 12.12)	0.13
C-reactive protein*	mg/L	3.91 (2.59 to 5.22)	<0.001
Serum albumin	g/L	-0.28 (-0.82 to 0.25)	0.30
Waist circumference	cm	0.13 (-0.003 to 0.25)	0.057
Systolic blood pressure	mmHg	0.09 (0.02 to 0.17)	0.019
Diastolic blood pressure	mmHg	0.17 (0.03 to 0.31)	0.016
Current smoking	yes vs. no	2.86 (-1.28 to 7.00)	0.18
Retinol intake*	mg/day	-0.57 (-3.21 to 2.08)	0.67
Ascorbic acid intake*	mg/day	-5.22 (-8.55 to -1.90)	0.002
Tocopherol intake*	mg/day	-2.30 (-6.03 to 1.43)	0.23
Micro-albuminuria	yes vs. no	-1.74 (-6.11 to 2.64)	0.44
Prior cardiovascular disease	yes vs. no	1.92 (-1.29 to 5.13)	0.24

Age-and sex-adjusted regression coefficients are expressed as change in MPO (in micrograms per liter) per unit increase of the variable under consideration. *Skewed variables were $\log_e$ transformed before analysis.

MPO and vascular function

Initial analyses showed that the relation between MPO and FMD was modified by an abnormal glucose metabolism status ($P_{interaction}$<0.05), but did not differ between subjects with impaired glucose metabolism and type 2 diabetes mellitus ($P_{interaction}$ = 0.50). Hence, the association between MPO and FMD was studied separately for the normal glucose metabolism group and the abnormal glucose metabolism group.

Brachial artery characteristics of the study population are shown in Table 3.

Table 3. Brachial artery characteristics according to glucose tolerance status.

	Normal glucose metabolism (*n*=188)	**Abnormal glucose metabolism (*n*=287)**	***P*-value**
Diameter (µm)			
Baseline	4520 (759)	4723 (741)	0.002
After FMD	4713 (749)	4888 (748)	0.008
After NMD	4952 (773)	5158 (733)	0.003
Absolute change in diameter (µm)			
After FMD	194 (128)	166 (162)	<0.001
After NMD	448 (197)	445 (223)	0.69
Percentage change in diameter (%)			
After FMD	4.49 (3.13)	3.62 (3.63)	<0.001
After NMD	10.31 (5.30)	9.85 (6.05)	0.21
Peak systolic velocity (cm/s)			
Baseline	56 (12)	59 (13)	0.007
After FMD	107 (29)	103 (22)	0.55
% Increase	94 (49)	81 (40)	0.005

Values are expressed as mean (SD). FMD, flow-mediated dilation; NMD, nitroglycerin-mediated dilation. Statistical significance was tested by Mann-Whitney analyses.

At baseline and after both NMD and FMD, the brachial artery diameter was larger in subjects with abnormal glucose metabolism compared with subjects with normal glucose metabolism. Absolute and relative changes in diameter after FMD, but not after NMD, were significantly smaller in individuals with abnormal glucose

metabolism. Peak systolic velocity was higher at baseline, whereas the relative increase after reactive hyperemia was lower, in the abnormal glucose metabolism group.

In the normal glucose metabolism group, mean FMD values did not significantly differ between subjects with MPO concentrations less than or greater than the median value (Figure 1). In contrast, FMD was significantly decreased at high MPO concentrations in the abnormal glucose metabolism group (Figure 1).

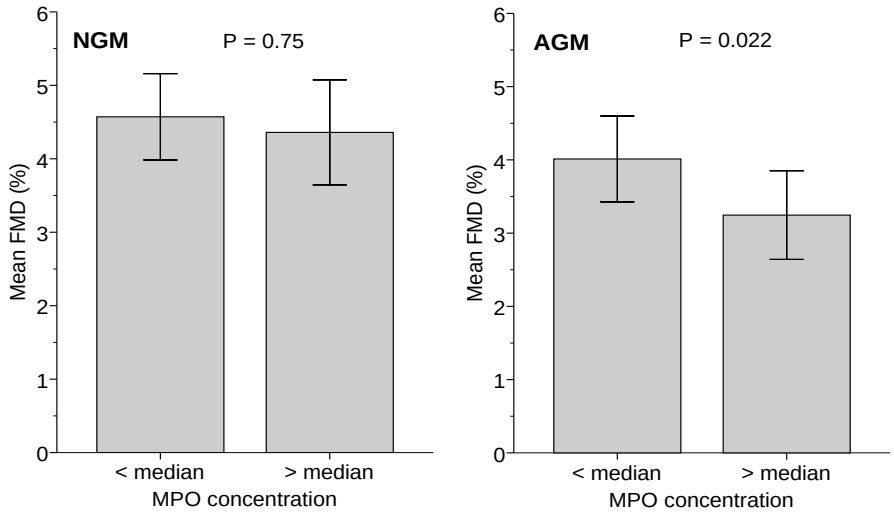

Figure 1. Flow-mediated dilation of the brachial artery according to low (less than the median [55.3 µg/L] of the entire cohort) or high (greater than the median) concentration of MPO in subjects with normal glucose metabolism (NGM) or abnormal glucose metabolism (AGM), that is, impaired glucose metabolism and type 2 diabetes mellitus diabetes. Flow-mediated dilation data are expressed as percentage change in brachial diameter and presented as means with 95% CI. Significance of differences was tested by Mann-Whitney analysis.

The association between MPO and FMD was studied with multivariable linear regression analyses (Table 4). A standard model including age, sex, peak flow velocity increase, and baseline diameter revealed a significant negative association between MPO and FMD in subjects with abnormal glucose metabolism (beta, −20.9 [95% confidence interval {CI}, −41.7 to −0.2] -µm change in FMD per 1-SD increment of MPO), but not in the normal glucose metabolism group (*P* = 0.83).

Table 4. Multivariable regression models for the relation between MPO and FMD (dependent variable) according to glucose tolerance status.

Model	Normal glucose metabolism		Abnormal glucose metabolism	
	Beta (95% CI)	***P*-value**	**Beta (95% CI)**	***P*-value**
Standard model	2.0 (-16.0 to 20.0)	0.83	-20.9 (-41.7 to -0.2)	0.048
Model 1: standard model + nitroglycerin-mediated dilation	0.2 (-16.0 to 16.5)	0.98	-31.1 (-50.0 to -12.3)	0.001
Model 2: model 1+ mean arterial pressure	0.8 (-15.4 to 17.0)	0.92	-28.8 (-47.9 to -9.7)	0.003
Model 2 + C-reactive protein*	1.8 (-15.0 to 18.6)	0.83	-25.4 (-45.2 to -5.6)	0.012
Model 2 + ascorbic acid intake*	0.6 (-16.1 to 17.3)	0.95	-25.6 (-44.5 to -6.6)	0.008
Model 2 + Framingham risk score	1.0 (-15.2 to 17.2)	0.91	-25.5 (-44.2 to -6.7)	0.008
Model 2 + waist circumference	0.2 (-15.9 to 16.3)	0.98	-26.8 (-46.0 to -7.6)	0.006
Model 2 + prior cardiovascular disease	0.9 (-15.5 to 17.2)	0.92	-28.9 (-48.1 to -9.6)	0.003
Model 2 + micro-albuminuria	1.0 (-15.2 to 17.2)	0.90	-28.0 (-47.0 to -8.9)	0.004

Regression coefficients are expressed as absolute change in diameter (in µm) per 1 SD (19.3 µg/L) increment of myeloperoxidase. Standard model: myeloperoxidase + age, sex, peak flow velocity increase, and baseline diameter. *Skewed variables were $\log_e$ transformed prior to linear regression analyses.

Myeloperoxidase and NMD were not significantly associated (P = 0.11), and this association was not modified by glucose metabolism status ($P_{interaction}$ = 0.27). In the group with abnormal glucose metabolism, the association between MPO and FMD remained significant after adjustment for endothelium-independent vasodilation by including NMD in the model (β,−31.1 [95% CI, −50.0 to −12.3]) (Table 4, model 1). Further adjustment for mean arterial pressure (Table 4, model 2) did not alter this association. In addition, none of the other variables tested (CRP, ascorbic acid intake, Framingham risk score, waist circumference, prior CVD, or microalbuminuria)

attenuated the association between MPO and FMD in individuals with abnormal glucose metabolism.

Accessory analyses

Because CRP and ascorbic acid intake were both independent correlates of MPO, both variables might, either directly or through their association with MPO, be associated with FMD. To gain more insight in these associations, we explored additional linear regression models that were stratified for glucose metabolism status and adjusted for age, sex, baseline diameter, peak flow velocity increase, mean arterial blood pressure, and NMD. In these models, MPO and CRP were negatively and ascorbic acid intake was positively associated with FMD in subjects with abnormal glucose metabolism, but not in individuals with normal glucose metabolism (Figure 2, open bars).

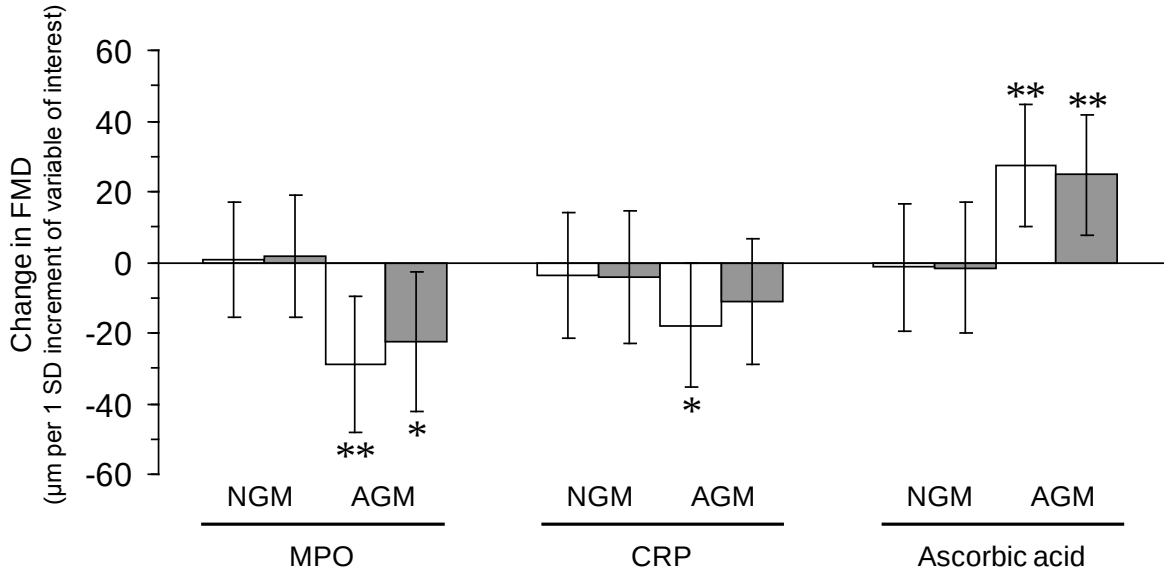

Figure 2. Changes in absolute FMD (95% CI) per SD increment in plasma concentrations of MPO, CRP, and ascorbic acid intake (the latter two after $\log_e$ transformation) in subjects with normal glucose metabolism (NGM) and abnormal glucose metabolism (AGM). Open bars represent the association of the variable of interest (MPO, CRP, or ascorbic acid consumption) with FMD, adjusted for age, sex, baseline diameter, pulse wave velocity increase, mean arterial blood pressure, and NMD. Solid bars represent the association of the variable of interest (MPO, CRP, or ascorbic acid) with FMD after additional adjustment for both other variables of interest. *P<0.05 and **P<0.01.

These associations were slightly attenuated after further mutual adjustment for the other variables of interest (MPO, CRP, and ascorbic acid intake). However, the

negative association between MPO and FMD as well as the positive association between ascorbic acid intake and FMD in subjects with abnormal glucose metabolism remained significant (Figure 2, solid bars). In contrast, mutual adjustment rendered the association between CRP and FMD nonsignificant.

Discussion

Myeloperoxidase plasma concentrations were elevated and FMD was decreased in subjects with an abnormal glucose metabolism. Myeloperoxidase was significantly inversely associated with endothelium-dependent vasodilation in individuals with abnormal glucose metabolism, but not in those with normal glucose metabolism.

Glucose metabolism parameters were associated with MPO concentrations. Furthermore, CRP, a marker of inflammation, was an independent correlate of MPO. These findings are in agreement with previous observations of increased inflammation in impaired glucose metabolism and type 2 diabetes mellitus,[26] and elevated levels of the MPO cosubstrate hydrogen peroxide at high glucose concentrations.[13] Hence, MPO, an enzyme that converts hydrogen peroxide to even more reactive compounds, may be particularly detrimental to the vascular endothelium in the abnormal glucose metabolism group. The role of vitamin consumption in preventing CVD and influencing plasma MPO concentrations has only partially been elucidated. Ascorbic acid can inhibit and reverse hypochlorous acid–induced chlorination of LDL in vitro [27] and protect against NO scavenging.[28] In the present study, a significant inverse independent association was found between MPO and intake of ascorbic acid. This association was not observed with both other vitamins.

Myeloperoxidase was inversely associated with FMD, independent of NMD, but only in subjects with abnormal glucose metabolism. Flow-mediated dilation reflects “total” vascular function, whereas NMD is thought to reflect the proportion of vascular function that does not depend on endothelium-derived vasodilators. None of the traditional risk factors, including microalbuminuria and prior CVD, altered the strength of the relation between MPO and FMD. Taken together, these results strongly suggest that MPO is an independent correlate of endothelium-dependent vascular function in persons with abnormal glucose metabolism.

A positive association between ascorbic acid and FMD was anticipated because it has been shown that ascorbic acid protects constituents of the artery wall from oxidation by HOCl.[29] In addition, improvement of FMD after both acute and chronic ascorbic acid administration has been reported in patients with coronary artery disease[30] and chronic heart failure [31] and in insulin resistant subjects and smokers,[32] although such an improvement was not found in healthy subjects. In line with these reports, we found a positive association between ascorbic acid intake and endothelium-dependent vascular function, but only in individuals with abnormal glucose metabolism. The negative association between ascorbic acid intake and MPO concentration in the present study suggests that lowering of MPO may be one of the routes by which ascorbic acid reduces oxidative stress and improves vascular function in subjects with abnormal glucose metabolism. On the other hand, ascorbic acid intake and MPO were both independent correlates of FMD and remained so after mutual adjustment, indicating that ascorbic acid may improve vascular function by both MPO-dependent and MPO-independent mechanisms.

C-reactive protein is known to stimulate polymorphonuclear cells to release MPO.[33] In the present study, CRP was, like ascorbic acid intake, an independent correlate of plasma MPO concentration and was associated with FMD in individuals with abnormal glucose metabolism. However, the association between CRP and FMD was weaker and less robust in comparison with the association between ascorbic acid intake and FMD, and lost significance after mutual adjustment. This is in agreement with previous studies in which associations between CRP and FMD were either non-significant[34] or modest and rendered nonsignificant after adjustment for traditional risk factors.[12] Both inflammation and oxidative stress may be involved in impairment of endothelium-dependent vascular function. The fact that MPO is a marker of both inflammation and oxidative stress, whereas CRP only reflects inflammation, may therefore explain the stronger and more robust association of MPO with FMD. Local release and retention of MPO in the vasculature, as contrasted to CRP that is mainly of hepatic origin, may also contribute to a stronger association of MPO with FMD.

Our study had some limitations. First, the study population was limited to elderly subjects; and because atherosclerosis progresses with age, the results may be different in other age groups. A second limitation is that vitamin intake was assessed by questionnaire, and it is conceivable that measurement of ascorbic acid

in plasma would have revealed a more pronounced association with MPO. Third, because MPO may be released from activated leukocytes in serum and heparin plasma during sample handling, MPO values differ considerably between serum, heparin-plasma, and EDTA-plasma.[35,36] EDTA-plasma was our choice because it may best reflect circulating concentrations of MPO. It should be noted, however, that measurement of MPO in serum or heparin-plasma or even whole blood would have provided other, but potentially equally valuable, information because it probably better reflects the total reservoir of MPO. Major strengths of our study include the large number of subjects and the availability of a wide array of clinical and biochemical variables to control for potential confounding.

In summary, in individuals with abnormal glucose metabolism, MPO was elevated and independently inversely associated with endothelium-dependent vasodilation.

References

1. Zhang R, Brennan ML, Fu X, Aviles RJ, Pearce GL, Penn MS, Topol EJ, Sprecher DL, Hazen SL. Association between myeloperoxidase levels and risk of coronary artery disease. *JAMA*. 2001;286:2136-2142.
2. Brennan ML, Penn MS, Van Lente F, Nambi V, Shishehbor MH, Aviles RJ, Goormastic M, Pepoy ML, McErlean ES, Topol EJ, Nissen SE, Hazen SL. Prognostic value of myeloperoxidase in patients with chest pain. *N Engl J Med*. 2003;349:1595-1604.
3. Baldus S, Rudolph V, Roiss M, Ito WD, Rudolph TK, Eiserich JP, Sydow K, Lau D, Szocs K, Klinke A, Kubala L, Berglund L, Schrepfer S, Deuse T, Haddad M, Risius T, Klemm H, Reichenspurner HC, Meinertz T, Heitzer T. Heparins increase endothelial nitric oxide bioavailability by liberating vessel-immobilized myeloperoxidase. *Circulation*. 2006;113:1871-1878.
4. Mocatta TJ, Pilbrow AP, Cameron VA, Senthilmohan R, Frampton CM, Richards AM, Winterbourn CC. Plasma concentrations of myeloperoxidase predict mortality after myocardial infarction. *J Am Coll Cardiol*. 2007;49:1993-2000.
5. Brevetti G, Schiano V, Laurenzano E, Giugliano G, Petretta M, Scopacasa F, Chiariello M. Myeloperoxidase, but not C-reactive protein, predicts cardiovascular risk in peripheral arterial disease. *Eur Heart J*. 2008;29:224-230.
6. Klebanoff SJ. Myeloperoxidase: friend and foe. *J Leukoc Biol*. 2005;77:598-625.
7. Miller RA, Britigan BE. Role of oxidants in microbial pathophysiology. *Clin Microbiol Rev*. 1997;10:1-18.
8. Wang Z, Nicholls SJ, Rodriguez ER, Kummu O, Horkko S, Barnard J, Reynolds WF, Topol EJ, DiDonato JA, Hazen SL. Protein carbamylation links inflammation, smoking, uremia and atherogenesis. *Nat Med*. 2007;13:1176-1184.
9. Schindhelm RK, Van der Zwan LP, Teerlink T, Scheffer PG. Myeloperoxidase: a useful biomarker for cardiovascular disease risk stratification? *Clin Chem*. 2009;55:1462-1470.
10. Shao B, Oda MN, Oram JF, Heinecke JW. Myeloperoxidase: an inflammatory enzyme for generating dysfunctional high density lipoprotein. *Curr Opin Cardiol*. 2006;21:322-328.
11. Abu-Soud HM, Hazen SL. Nitric oxide is a physiological substrate for mammalian peroxidases. *J Biol Chem*. 2000;275:37524-37532.
12. Vita JA, Brennan ML, Gokce N, Mann SA, Goormastic M, Shishehbor MH, Penn MS, Keaney JF, Jr., Hazen SL. Serum myeloperoxidase levels independently predict endothelial dysfunction in humans. *Circulation*. 2004;110:1134-1139.
13. Zhang C, Yang J, Jennings LK. Leukocyte-derived myeloperoxidase amplifies high-glucose--induced endothelial dysfunction through interaction with high-glucose--stimulated, vascular non--leukocyte-derived reactive oxygen species. *Diabetes*. 2004;53:2950-2959.
14. Guzik TJ, Mussa S, Gastaldi D, Sadowski J, Ratnatunga C, Pillai R, Channon KM. Mechanisms of increased vascular superoxide production in human diabetes mellitus: role of NAD(P)H oxidase and endothelial nitric oxide synthase. *Circulation*. 2002;105:1656-1662.

15. Mooy JM, Grootenhuis PA, De Vries H, Valkenburg HA, Bouter LM, Kostense PJ, Heine RJ. Prevalence and determinants of glucose intolerance in a Dutch caucasian population. The Hoorn Study. *Diabetes Care*. 1995;18:1270-1273.
16. Spijkerman AM, Adriaanse MC, Dekker JM, Nijpels G, Stehouwer CD, Bouter LM, Heine RJ. Diabetic patients detected by population-based stepwise screening already have a diabetic cardiovascular risk profile. *Diabetes Care*. 2002;25:1784-1789.
17. Liu PY, Liu YW, Lin LJ, Chen JH, Liao JK. Evidence for statin pleiotropy in humans: differential effects of statins and ezetimibe on rho-associated coiled-coil containing protein kinase activity, endothelial function, and inflammation. *Circulation*. 2009;119:131-138.
18. Alberti KG, Zimmet PZ. Definition, diagnosis and classification of diabetes mellitus and its complications. Part 1: diagnosis and classification of diabetes mellitus provisional report of a WHO consultation. *Diabet Med*. 1998;15:539-553.
19. Schindhelm RK, Alssema M, Diamant M, Teerlink T, Dekker JM, Kok A, Kostense PJ, Nijpels G, Heine RJ, Scheffer PG. Comparison of two consecutive fat-rich and carbohydrate-rich meals on postprandial myeloperoxidase response in women with and without type 2 diabetes mellitus. *Metabolism*. 2008;57:262-267.
20. Grooteman MP, Gritters M, Wauters IM, Schalkwijk CG, Stam F, Twisk J, Ter Wee PM, Nube MJ. Patient characteristics rather than the type of dialyser predict the variability of endothelial derived surface molecules in chronic haemodialysis patients. *Nephrol Dial Transplant*. 2005;20:2751-2758.
21. Van der Zwan LP, Teerlink T, Dekker JM, Henry RM, Stehouwer CD, Jakobs C, Heine RJ, Scheffer PG. Circulating oxidized LDL: determinants and association with brachial flow-mediated dilation. *J Lipid Res*. 2008.
22. Corretti MC, Anderson TJ, Benjamin EJ, Celermajer D, Charbonneau F, Creager MA, Deanfield J, Drexler H, Gerhard-Herman M, Herrington D, Vallance P, Vita J, Vogel R. Guidelines for the ultrasound assessment of endothelial-dependent flow-mediated vasodilation of the brachial artery: a report of the International Brachial Artery Reactivity Task Force. *J Am Coll Cardiol*. 2002;39:257-265.
23. Henry RM, Ferreira I, Kostense PJ, Dekker JM, Nijpels G, Heine RJ, Kamp O, Bouter LM, Stehouwer CD. Type 2 diabetes is associated with impaired endothelium-dependent, flow-mediated dilation, but impaired glucose metabolism is not; The Hoorn Study. *Atherosclerosis*. 2004;174:49-56.
24. Wilson PW, D'Agostino RB, Levy D, Belanger AM, Silbershatz H, Kannel WB. Prediction of coronary heart disease using risk factor categories. *Circulation*. 1998;97:1837-1847.
25. Ocke MC, Bueno-de-Mesquita HB, Pols MA, Smit HA, van Staveren WA, Kromhout D. The Dutch EPIC food frequency questionnaire. II. Relative validity and reproducibility for nutrients. *Int J Epidemiol*. 1997;26 Suppl 1:S49-S58.
26. Muntner P, He J, Chen J, Fonseca V, Whelton PK. Prevalence of non-traditional cardiovascular disease risk factors among persons with impaired fasting glucose, impaired glucose tolerance,

diabetes, and the metabolic syndrome: analysis of the Third National Health and Nutrition Examination Survey (NHANES III). *Ann Epidemiol*. 2004;14:686-695.

27. Carr AC, McCall MR, Frei B. Oxidation of LDL by myeloperoxidase and reactive nitrogen species: reaction pathways and antioxidant protection. *Arterioscler Thromb Vasc Biol*. 2000;20:1716-1723.
28. Heller R, Werner-Felmayer G, Werner ER. Antioxidants and endothelial nitric oxide synthesis. *Eur J Clin Pharmacol*. 2005;62:21-28.
29. Jenner AM, Ruiz JE, Dunster C, Halliwell B, Mann GE, Siow RC. Vitamin C protects against hypochlorous Acid-induced glutathione depletion and DNA base and protein damage in human vascular smooth muscle cells. *Arterioscler Thromb Vasc Biol*. 2002;22:574-580.
30. Gokce N, Keaney JF, Jr., Frei B, Holbrook M, Olesiak M, Zachariah BJ, Leeuwenburgh C, Heinecke JW, Vita JA. Long-term ascorbic acid administration reverses endothelial vasomotor dysfunction in patients with coronary artery disease. *Circulation*. 1999;99:3234-3240.
31. Ellis GR, Anderson RA, Lang D, Blackman DJ, Morris RH, Morris-Thurgood J, McDowell IF, Jackson SK, Lewis MJ, Frenneaux MP. Neutrophil superoxide anion--generating capacity, endothelial function and oxidative stress in chronic heart failure: effects of short- and long-term vitamin C therapy. *J Am Coll Cardiol*. 2000;36:1474-1482.
32. Hirai N, Kawano H, Hirashima O, Motoyama T, Moriyama Y, Sakamoto T, Kugiyama K, Ogawa H, Nakao K, Yasue H. Insulin resistance and endothelial dysfunction in smokers: effects of vitamin C. *Am J Physiol Heart Circ Physiol*. 2000;279:H1172-H1178.
33. Singh U, Devaraj S, Jialal I. C-reactive protein stimulates myeloperoxidase release from polymorphonuclear cells and monocytes: implications for acute coronary syndromes. *Clin Chem*. 2009;55:361-364.
34. Lind L, Siegbahn A, Hulthe J, Elmgren A. C-reactive protein and e-selectin levels are related to vasodilation in resistance, but not conductance arteries in the elderly: the prospective investigation of the Vasculature in Uppsala Seniors (PIVUS) study. *Atherosclerosis*. 2008;199:129-137.
35. Chang PY, Wu TL, Hung CC, Tsao KC, Sun CF, Wu LL, Wu JT. Development of an ELISA for myeloperoxidase on microplate: normal reference values and effect of temperature on specimen preparation. *Clin Chim Acta*. 2006;373:158-163.
36. Scheffer PG, Van der Zwan LP, Schindhelm RK, Vermue HP, Teerlink T. Myeloperoxidase concentrations in EDTA-plasma of healthy subjects are discordant with concentrations in heparin-plasma and serum. *Clin Biochem*. 2009;42:1490-1492.

Chapter 6A

Hyperglycemia and oxidative stress strengthen the association between myeloperoxidase and blood pressure

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Abstract

Scavenging of the vasodilator nitric oxide by myeloperoxidase activity in the vasculature may contribute to hypertension. Because hydrogen peroxide is a cosubstrate of myeloperoxidase, hyperglycemia-induced oxidative stress may strengthen the relationship between myeloperoxidase and blood pressure. We investigated this relationship and its modification by hyperglycemia and oxidative stress in a population-based cohort of elderly subjects with normal glucose metabolism (n=267), impaired glucose metabolism (n=189), and type 2 diabetes (n=290). In an age- and sex-adjusted linear regression model, plasma myeloperoxidase was positively associated with systolic blood pressure (2.10 mm Hg per 1 SD increment of myeloperoxidase [95% CI: 0.66 to 3.54]), and this association was stronger at higher levels of fasting glucose (0.61 [-1.70 to 2.93], 1.33 [-1.43 to 4.10], and 3.42 [1.01 to 5.82] for increasing tertiles of glucose) and higher plasma levels of oxidized low-density lipoprotein (0.92 [-1.31 to 3.14], 2.00 [-0.71 to 4.70], and 3.58 [0.98 to 6.19] for increasing tertiles of oxidized low-density lipoprotein). Likewise, the relationship between myeloperoxidase and blood pressure was strongest under conditions associated with oxidative stress, like obesity, low high-density lipoprotein cholesterol, metabolic syndrome, and type 2 diabetes. The strength of these associations was only marginally attenuated by adjustment for other cardiovascular risk factors. Our data demonstrate that myeloperoxidase is positively and independently associated with blood pressure, and this association is strongest in subjects with (hyperglycemia-induced) oxidative stress. These observations, together with emerging evidence that myeloperoxidase-derived oxidants contribute to the initiation and propagation of cardiovascular disease, identify myeloperoxidase as a promising target for drug development.

Introduction

Increasing evidence suggests that inflammation plays a role in the development of hypertension.[1] Myeloperoxidase (MPO) is an enzyme linked to both inflammation and oxidative stress, but the relationship between MPO and blood pressure remains largely untested. Release of MPO by neutrophils and monocytes during inflammation plays an important role in the innate immune response,[2] but MPO activity may also lead to tissue damage and precipitate atherogenesis.[3] Many studies have demonstrated that systemic MPO levels predict risk throughout the spectrum of cardiovascular diseases.[4-8] MPO catalyzes the production of hypochlorous acid and a range of other highly reactive species. These MPO-derived reactive substances may damage the arterial wall, thereby reducing its elasticity. In addition, by several mechanisms, MPO reduces the bioavailability of the endogenous vasodilator nitric oxide.[3,9] Together, these mechanisms may lead to an increase in blood pressure. Because hydrogen peroxide is an obligate cosubstrate of MPO, the activity of MPO in the vasculature may be enhanced by increased local production of reactive oxygen species. Vascular production of superoxide and its dismutation product hydrogen peroxide has been shown to be stimulated by high glucose concentrations, resulting in increased activity of MPO.[10] We therefore hypothesize that the relationship between MPO and blood pressure is stronger on a background of (hyperglycemia-induced) oxidative stress.

The aims of our study were to assess the relationship between plasma levels of MPO and blood pressure and to test the hypothesis that this relationship is strengthened by (hyperglycemia-induced) oxidative stress.

Methods

Subjects

The present study was conducted in the Hoorn Study[11] follow-up examination conducted in 2000 and the Hoorn Screening Study,[12] both of which are population-based studies in a white population. From the 822 participants, we excluded subjects with missing data on primary variables of interest, leaving 746 subjects, of whom 267 had a normal glucose metabolism, 189 had an impaired glucose metabolism, and 290 had type 2 diabetes, according to WHO 1999 criteria.[13] The local ethics

committee approved the study, and all participants gave their written informed consent.

Blood pressure measurement

Subjects were in a sitting position and had rested for 5 minutes before measurement of systolic (SBP) and diastolic (DBP) blood pressure. A random-zero sphygmomanometer (Hawksley-Gelman, Lansing, Sussex, United Kingdom) was used for duplicate measurements, and mean values were used in analyses. Hypertension was defined as SBP >140 mm Hg or DBP >90 mm Hg, according to criteria described in the seventh report of the Joint National Committee on Prevention, Detection, Evaluation, and Treatment of High Blood Pressure.[14]

Biochemical analyses

A sandwich enzyme-linked immunosorbent assay (Mercodia, Uppsala, Sweden) was used to determine MPO concentrations in EDTA-plasma, with intra- and interassay coefficients of variation of 3.9% and 5.0%, respectively.[15] Plasma C-reactive protein (CRP) concentrations were determined with a highly sensitive in-house sandwich enzyme-linked immunosorbent assay.[16] Circulating plasma oxidized low-density lipoprotein (LDL) was determined by competitive enzyme-linked immunosorbent assay (Mercodia, Uppsala, Sweden).[17] Hemoglobin A1c (HbA1c) was analyzed by ion-exchange high-performance liquid chromatography (reference range, 4.3 to 6.1%) on a modular monitoring system (Bio-Rad, Veenendaal, The Netherlands). Glucose was measured enzymatically (Roche, Mannheim, Germany), and insulin was determined by a 2-site immunoradiometric assay (Medgenix Diagnostics, Fleurus, Belgium). Total and high-density lipoprotein (HDL) cholesterol and triglycerides were measured by standard enzymatic methods (Roche). LDL-cholesterol concentration was determined with a direct method by the “N-geneous” assay (GenZyme, Cambridge, Mass). With this method, a triglyceride concentration up to 13.5 mmol/L does not interfere with measurement of LDL-cholesterol.

Microalbuminuria, metabolic syndrome, and cardiovascular disease

Microalbuminuria was defined as urinary albumin/creatinine ratio ≥2.0 mg/mmol. Metabolic syndrome was defined according to the Adult Treatment Panel

III of the National Cholesterol Education Program, ie, 3 or more of the following: fasting glucose ≥6.1 mmol/L, HDL-cholesterol <1.03 mmol/L (men) or <1.29 mmol/L (women), triglycerides ≥1.7 mmol/L, waist circumference >102 cm (men) or >88 cm (women), and blood pressure ≥130/85 mm Hg.[18] Prior cardiovascular disease was defined as Minnesota Code 1.1 to 1.3, 4.1 to 4.3, 5.1 to 5.3, or 7.1 on the ECG[19]; coronary bypass operation or angioplasty; an ankle-brachial blood pressure index <0.9 in either leg; peripheral arterial bypass; or amputation for atherosclerotic disease.

Statistics

Data are presented as mean and SD or, if skewed, median and interquartile range. Bivariate associations were assessed by calculation of the Spearman ρ. Skewed variables were natural-log transformed before trend analyses and linear regression analyses. Statistical significance for linear trend across tertiles of MPO was calculated by linear regression, with adjustment for age and sex, or by linear-by-linear X^2-tests. Multivariable linear regression analysis was used to assess the relationship between (log-transformed) MPO and blood pressure. Confounding by other risk factors was investigated by adding these factors one by one to age- and sex-adjusted models and by investigating fully adjusted models including all risk factors. Regression coefficients were expressed as change in blood pressure (mm Hg) per SD increase of log-transformed MPO. Because the variance of MPO did not significantly differ (tested with the Levene test for homogeneity of variance) between the various strata investigated, the SD of the entire population was used for all stratified analyses. Data were analyzed using SPSS software, version 15 (SPSS Inc., Chicago, IL). A 2-tailed probability value <0.05 was considered to indicate statistical significance.

Results

Population characteristics

Of the 746 study participants 432 (58%) were hypertensive. After adjustment for age and sex, it was found that blood pressure, prevalence of hypertension, and use of antihypertensive medication significantly increased across tertiles of MPO (Table 1).

Table 1. Subject characteristics by tertiles of MPO.

		MPO			
		1st tertile	2nd tertile	3rd tertile	
Variable	**Unit**	**<50.6 µg/L**	**50.6-62.7 µg/L**	**>62.7 µg/L**	***P*-value**
N		248	249	249	
Age	years	68.0 (7.1)	68.6 (6.9)	69.9 (7.4)	0.007
Sex, male	%	56	46	47	0.054
Systolic blood pressure	mmHg	140 (19)	141 (21)	146 (21)	0.003
Diastolic blood pressure	mmHg	82 (10)	83 (11)	84 (11)	0.009
Hypertension	%	53	56	64	0.016
C-reactive protein*	mg/L	1.6 (0.9-2.8)	2.0 (1.1-4.2)	3.6 (1.7-6.9)	<0.001
HbA1c	%	6.0 (0.7)	6.1 (0.7)	6.2 (0.9)	<0.001
Fasting glucose*	mmol/L	6.0 (5.5-6.9)	6.0 (5.4-6.9)	6.3 (5.7-7.3)	<0.001
Insulin*	pmol/L	62 (44-84)	59 (41-86)	68 (46-101)	0.01
T2DM	%	38	35	44	0.18
IGM	%	25	25	26	0.94
Body mass index	kg/m^2	27.1 (3.6)	27.9 (4.3)	28.2 (4.7)	0.005
Waist circumference	cm	95.1 (11.1)	96.7 (12.5)	97.7 (13.1)	0.002
LDL-cholesterol	mmol/L	3.6 (1.0)	3.6 (0.9)	3.6 (0.9)	0.39
HDL-cholesterol	mmol/L	1.34 (0.38)	1.43 (0.42)	1.37 (0.40)	0.97
Triglycerides*	mmol/L	1.4 (1.0-1.8)	1.3 (1.0-1.9)	1.4 (1.0-1.9)	0.33
Oxidized LDL	U/L	65.4 (15.6)	63.9 (14.5)	65.0 (15.5)	0.77
Micro-albuminuria	%	13	15	18	0.16
Prior cardiovascular disease	%	46	46	55	0.048
Metabolic syndrome	%	42	43	50	0.052
Current smoker	%	10	20	16	0.053
Anti-hypertensive med.	%	36	36	47	0.012
Lipid lowering medication	%	17	18	18	0.89

T2DM indicates type 2 diabetes; IGM, impaired glucose metabolism. *Skewed variables were transformed by natural logarithm before trend analyses. *P* values represent trends across tertiles and were obtained by age- and sex-adjusted linear regression analysis or, for dichotomous variables, by X^2 linear-by-linear analyses.

Higher tertiles of MPO were associated with higher CRP levels, and also, if MPO was analyzed as a continuous variable, a significant association with CRP was observed (ρ=0.29; *P*<0.001). HbA1c, fasting glucose, and insulin levels also increased in parallel with MPO, but the percentage of individuals with type 2 diabetes and impaired glucose metabolism was not significantly different between MPO tertiles. Body mass index (BMI), waist circumference, and prior cardiovascular disease significantly increased with higher MPO levels, but no significant relationships with lipid levels (including oxidized LDL) or the use of lipid-lowering medication were observed.

Association between MPO and blood pressure

Linear regression analysis was used to investigate the relationship between MPO levels and SBP (Table 2) and DBP (Table 3). In the entire cohort, MPO was significantly associated with SBP and DBP, both in crude analysis and after adjustment for age and sex. Additional adjustment of the age- and sex-adjusted models for a range of potentially confounding risk factors, including use of antihypertensive medication and CRP, only marginally altered the strength of these associations.

Table 2. Multivariable linear regression models of the relation between MPO and SBP in strata of fasting glucose.

Model	Overall		1st tertile glucose <5.8 mmol/L		2nd tertile glucose 5.8-6.5 mmol/L		3rd tertile glucose >6.5 mmol/L	
	β (95%CI)	*P*	β (95%CI)	*P*	β (95%CI)	*P*	β (95%CI)	*P*
Crude	2.53 (1.06 to 3.99)	<0.001	1.04 (-1.38 to 3.46)	0.40	1.88 (-0.83 to 4.59)	0.17	3.82 (1.39 to 6.26)	0.002
Model 1: MPO* + age + sex	2.10 (0.66 to 3.54)	0.004	0.61 (-1.70 to 2.93)	0.60	1.33 (-1.43 to 4.10)	0.34	3.42 (1.01 to 5.82)	0.006
Model 1 + CRP*	1.69 (0.21 to 3.17)	0.025	-0.03 (-2.40 to 2.34)	0.98	1.36 (-1.45 to 4.17)	0.34	3.31 (0.84 to 5.78)	0.009
Model 1 + glucose*	1.71 (0.28 to 3.14)	0.019	0.43 (-1.87 to 2.73)	0.71	1.32 (-1.45 to 4.10)	0.35	3.39 (0.96 to 5.82)	0.006
Model 1 + body mass index	1.83 (0.40 to 3.25)	0.012	1.01 (-1.21 to 3.23)	0.37	1.03 (-1.74 to 3.79)	0.47	3.37 (0.95 to 5.79)	0.006
Model 1 + triglycerides*	2.12 (0.69 to 3.55)	0.004	0.64 (-1.69 to 2.96)	0.59	1.32 (-1.45 to 4.08)	0.35	3.48 (1.09 to 5.87)	0.005
Model 1 + microalbuminuria	2.05 (0.61 to 3.50)	0.005	0.57 (-1.74 to 2.88)	0.63	1.23 (-1.55 to 4.01)	0.39	3.44 (1.02 to 5.86)	0.005
Model 1 + prior cardiovascular disease	2.05 (0.57 to 3.53)	0.007	0.59 (-1.76 to 2.94)	0.63	1.35 (-1.48 to 4.17)	0.35	3.33 (0.83 to 5.83)	0.009
Model 1 + current smoker	2.28 (0.84 to 3.73)	0.002	0.80 (-1.51 to 3.11)	0.50	1.37 (-1.38 to 4.13)	0.33	3.55 (1.09 to 6.01)	0.005
Model 1 + anti-hypertensive medication	1.88 (0.44 to 3.31)	0.010	0.47 (-1.81 to 2.75)	0.69	1.22 (-1.56 to 3.99)	0.39	3.32 (0.91 to 5.73)	0.007
Model 1 + lipid lowering medication	2.10 (0.65 to 3.55)	0.005	0.61 (-1.71 to 2.92)	0.61	1.32 (-1.46 to 4.11)	0.35	3.38 (0.96 to 5.80)	0.006
Fully adjusted model†	1.61 (0.13 to 3.10)	0.033	0.26 (-2.10 to 2.61)	0.83	1.11 (-1.77 to 4.00)	0.45	3.50 (0.86 to 6.14)	0.010

β values represent increase in systolic blood pressure (mm Hg) per SD increase of MPO. *Skewed variables were transformed by natural logarithm. †Adjusted for age, sex, CRP, glucose, BMI, triglycerides, microalbuminuria, prior cardiovascular disease, current smoking, and use of antihypertensive and lipid-lowering medication.

Table 3. Multivariable linear regression models of the relation between MPO and DBP in strata of fasting glucose.

Model	Overall		1st tertile glucose <5.8 mmol/L		2nd tertile glucose 5.8-6.5 mmol/L		3rd tertile glucose >6.5 mmol/l	
	β (95%CI)	*P*	β (95%CI)	*P*	β (95%CI)	*P*	β (95%CI)	*P*
Crude	0.85 (0.07 to 1.63)	0.033	0.09 (-1.25 to 1.43)	0.90	0.26 (-1.21 to 1.73)	0.73	1.66 (0.45 to 2.87)	0.008
Model 1: MPO* + age + sex	0.98 (0.20 to 1.76)	0.014	0.06 (-1.29 to 1.40)	0.93	0.60 (-0.89 to 2.10)	0.43	1.76 (0.54 to 2.98)	0.005
Model 1 + CRP*	0.83 (0.03 to 1.63)	0.043	-0.16 (-1.54 to 1.23)	0.83	0.71 (-0.81 to 2.23)	0.36	1.67 (0.42 to 2.93)	0.009
Model 1 + glucose*	0.77 (0.00 to 1.55)	0.051	0.00 (-1.35 to 1.35)	1.00	0.60 (-0.90 to 2.10)	0.43	1.67 (0.45 to 2.90)	0.008
Model 1 + body mass index	0.79 (0.03 to 1.55)	0.042	0.28 (-1.01 to 1.58)	0.67	0.28 (-1.18 to 1.74)	0.70	1.68 (0.46 to 2.90)	0.007
Model 1 + triglycerides*	0.99 (0.22 to 1.77)	0.012	0.03 (-1.32 to 1.38)	0.96	0.57 (-0.90 to 2.04)	0.45	1.78 (0.57 to 3.00)	0.004
Model 1 + microalbuminuria	0.94 (0.16 to 1.72)	0.018	0.01 (-1.33 to 1.35)	0.98	0.57 (-0.94 to 2.08)	0.46	1.78 (0.57 to 3.00)	0.004
Model 1 + prior cardiovascular disease	0.97 (0.17 to 1.77)	0.017	0.05 (-1.32 to 1.42)	0.94	0.57 (-0.96 to 2.09)	0.47	1.73 (0.46 to 3.00)	0.008
Model 1 + current smoker	1.08 (0.30 to 1.86)	0.007	0.20 (-1.14 to 1.53)	0.77	0.61 (-0.89 to 2.12)	0.42	1.90 (0.66 to 3.14)	0.003
Model 1 + anti-hypertensive medication	0.87 (0.09 to 1.64)	0.029	0.00 (-1.34 to 1.33)	0.99	0.52 (-0.97 to 2.02)	0.49	1.72 (0.49 to 2.94)	0.006
Model 1 + lipid lowering medication	0.98 (0.20 to 1.76)	0.014	0.05 (-1.30 to 1.39)	0.95	0.60 (-0.91 to 2.10)	0.43	1.76 (0.54 to 2.99)	0.005
Fully adjusted model†	0.81 (0.02 to 1.61)	0.045	0.07 (-1.30 to 1.43)	0.92	0.43 (-1.06 to 1.93)	0.57	1.82 (0.49 to 3.16)	0.008

β values represent increase in diastolic blood pressure (mm Hg) per SD increase of MPO. *Skewed variables were transformed by natural logarithm. †Adjusted for age, sex, CRP, glucose, BMI, triglycerides, microalbuminuria, prior cardiovascular disease, current smoking, and use of antihypertensive and lipid-lowering medication.

Modification of the relationship between MPO and blood pressure by glucose

Because we hypothesized that the adverse impact of MPO on blood pressure might be enhanced by hyperglycemia, we also examined the relationship between MPO and blood pressure in strata of fasting glucose. In individuals with glucose levels in the lowest tertile, MPO was not significantly associated with SBP (β [95% CI] of 1.04 [-1.38 to 3.46] mm Hg per 1-SD increase of MPO), but this association was stronger in individuals with intermediate glucose levels (1.88 [-0.83 to 4.59]) and most prominent in individuals with fasting glucose in the highest tertile (3.82 [1.39 to 6.26]; Table 2). A similar trend across tertiles of fasting glucose was observed for the relationship between MPO and DBP (Table 3). The associations of MPO with both SBP and DBP were hardly influenced by adjustment for age, sex, and potentially confounding risk factors. Notably, in subjects with glucose in the highest tertile, only MPO and age contributed significantly to the fully adjusted models for SBP and DBP.

In support of the effect of fasting glucose on the association between MPO and blood pressure, the age- and sex-adjusted association between MPO and SBP was weak and nonsignificant in subjects with normal or impaired glucose metabolism (β=0.48, *P*=0.70, and β=1.36, *P*=0.33, respectively) but much stronger and highly significant in subjects with type 2 diabetes (β=3.35; *P*=0.004) (Figure 1). Likewise, the relationship between MPO and DBP was nonsignificant in subjects with normal glucose metabolism (β=-0.06; *P*=0.94) but stronger and significant in subjects with impaired glucose metabolism or type 2 diabetes (β=1.75, *P*=0.027, and β=1.18, *P*=0.047, respectively) (Figure 1).

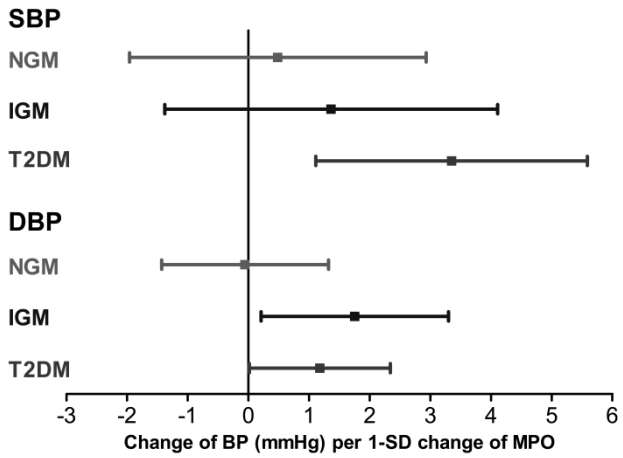

Figure 1. Effect of glucose metabolism on the relationship between MPO and blood pressure. The relationships between plasma concentrations of MPO and SBP and DBP in individuals with normal glucose metabolism (NGM), impaired glucose metabolism (IGM), and type 2 diabetes (T2DM) were obtained by age- and sex-adjusted linear regression analyses. Regression coefficients (95% CIs) are expressed as increase in blood pressure (mm Hg) per 1 SD increase of log-transformed MPO.

To further clarify whether the relationship between MPO and blood pressure was strengthened by hyperglycemia or by insulin resistance, we also examined the association between MPO and SBP in strata of HbA1c and insulin. The age- and sex-adjusted association was weak and non-significant in the lowest tertile of HbA1c (β [95% CI] of 0.26 [-2.15 to 2.68]) but much stronger for the intermediate and highest HbA1c tertiles (3.01 [0.30 to 5.71] and 2.66 [0.17 to 5.16], respectively). In contrast, the association between MPO and SBP was only marginally influenced by levels of insulin (age- and sex-adjusted β values of 1.37 [-1.45 to 4.18], 1.62 [-0.68 to 3.91], and 2.38 [-0.11 to 4.88] for increasing tertiles of insulin).

Influence of oxidative stress on the relationship between MPO and blood pressure

The fact that the relationship between MPO and blood pressure was strongest in the presence of high levels of glucose and that oxidized LDL is associated with glucose (ρ=0.11; *P*=0.003) is in accordance with the hypothesis that MPO activity is enhanced by (glucose-induced) oxidative stress, but it does not exclude involvement of non-oxidative effects of glucose. To gain better insight in the effect of oxidative stress on the relationship between MPO and blood pressure, we repeated the analyses after stratification of the cohort according to levels of oxidized LDL, a

validated marker of oxidative stress. The age- and sex-adjusted relationship between MPO and SBP by tertiles of oxidized LDL revealed a pattern similar to that observed for glucose, with the strongest association observed in individuals with high levels of oxidized LDL, ie, the third tertile (β=0.92 [-1.31 to 3.14], 2.00 [-0.71 to 4.70], and 3.58 [0.98 to 6.19] for increasing tertiles of oxidized LDL; Figure 2). Next, we examined whether the relationship between MPO and SBP was also modified by clinical or biochemical variables generally assumed to be associated with increased oxidative stress, ie, low HDL-cholesterol, obesity, and the metabolic syndrome. The concentration of HDL-cholesterol was inversely associated with oxidized LDL (ρ=-0.25; *P*<0.001), whereas BMI was positively associated with oxidized LDL (ρ=0.13; *P*<0.001). The association between MPO and SBP was strongest in subjects with HDL-cholesterol levels in the lowest tertile (β=3.96 [1.52 to 6.39]) and in subjects with a BMI in the highest tertile (β=3.52 [1.19 to 5.86] (Figure 2).

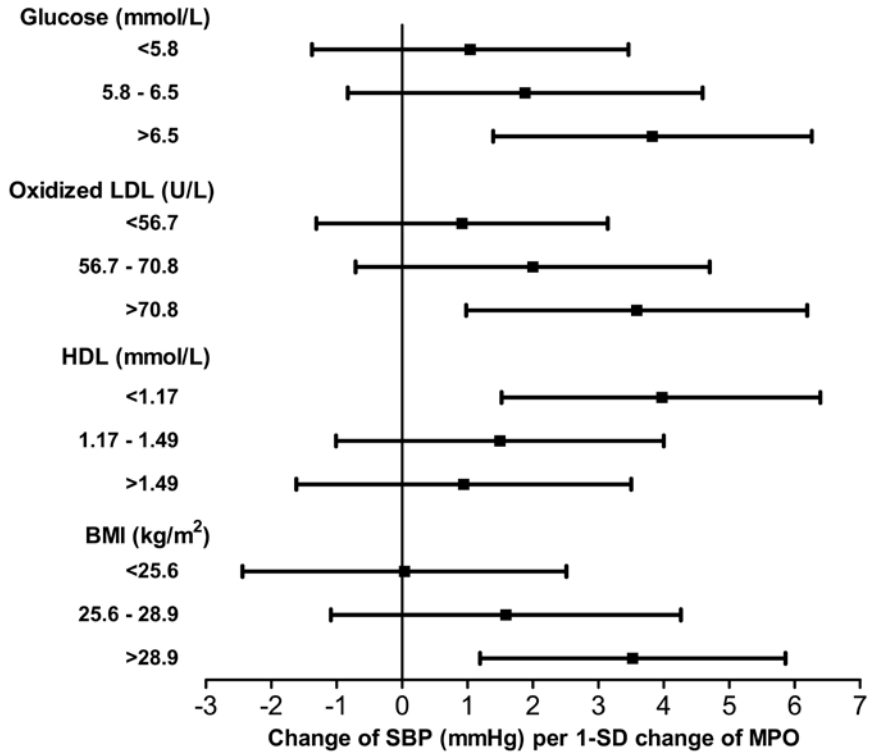

Figure 2. Impact of BMI and levels of fasting glucose, oxidized LDL, and HDL-cholesterol on the relationship between MPO and SBP. The relationships between plasma MPO and SBP were obtained by age- and sex-adjusted linear regression analyses after stratification by tertiles of the variable of interest. Regression coefficients (95% CIs) are expressed as increase in blood pressure (mm Hg) per 1 SD increase of log-transformed MPO.

Levels of oxidized LDL were higher in individuals with the metabolic syndrome compared to those without the metabolic syndrome (69.9±16.1 and 60.6±13.1 U/L, respectively; *P*<0.001), and in the former group, the age- and sex-adjusted association between MPO and blood pressure was strongest (β=2.47 [0.48 to 4.46] versus 1.17 [-0.75 to 3.08] for SBP and β=1.64 [0.58 to 2.69] versus 0.06 [-0.99 to 1.11] for DBP).

The interaction between the effects of BMI and MPO on SBP as shown in Figure 3 illustrates the clinical relevance of the MPO-associated increase in blood pressure, especially in subjects with a high BMI. The mean difference in SBP between individuals with both BMI and MPO in the highest tertile and individuals with both variables in the lowest tertile was 14 mm Hg.

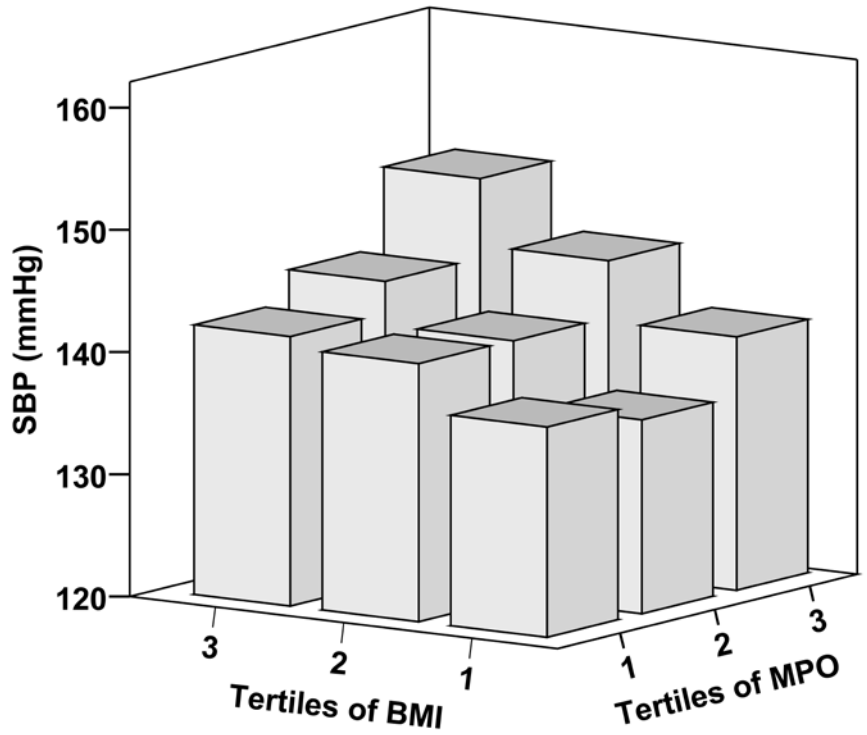

Figure 3. Combined effect of obesity and MPO on blood pressure. Bars represent unadjusted mean values of SBP after stratification according to tertiles of BMI and tertiles of plasma MPO.

Discussion

The main finding of the current study is that the concentration of MPO in the circulation was positively associated with both SBP and DBP. This association was independent of traditional cardiovascular risk factors, including CRP, and use of antihypertensive medication and was most prominent on a background of hyperglycemia or oxidative stress.

Potential mechanisms linking MPO to blood pressure

Local release by resident macrophages and transcytosis of MPO produced by activated neutrophils that are attracted and bound to sites of damaged endothelium are both sources of MPO in the vascular wall. The microenvironment of the subendothelial space of the vascular wall is especially conducive to MPO activity. Mitochondrial respiration, NAD(P)H oxidases, xanthine oxidase, and uncoupled nitric oxide synthase (NOS) are major sources of the highly reactive superoxide radical, which is actively converted into hydrogen peroxide by superoxide dismutase. Although less reactive than the superoxide radical, hydrogen peroxide is the cosubstrate for all MPO-catalyzed reactions. MPO amplifies the oxidative potential of hydrogen peroxide by producing a variety of reactive oxidants, including hypochlorous acid.[9]

Nitric oxide (NO), produced by endothelial NOS, is a powerful vasodilator and as such plays a critical role in the regulation of vascular tone. There are strong indications that MPO, by several mechanisms, may reduce the bioavailability of NO. First, NO serves as a substrate for peroxidases, and MPO may thus serve as a catalytic sink for NO.[20,21] Second, scavenging of NO by MPO-derived reactive substances may further reduce the bioavailability of NO. Third, hypochlorous acid can react with nitrogen atoms of the NOS substrate arginine to produce chlorinated arginine species that are inhibitors of all isoforms of NOS and have been shown to impair endothelium-dependent relaxation of rat aortic rings.[22] Finally, it has been demonstrated that hypochlorous acid is a potent inducer of uncoupling of endothelial NOS, thereby turning NOS into a superoxide producing enzyme.[23] Overall, reduction of NO bioavailability by MPO activity is a plausible mechanism for the adverse effect of MPO on blood pressure. In addition, MPO-derived reactive substances may

damage the arterial wall and reduce its elasticity, thereby contributing to hypertension.

Enhancement of the relationship between MPO and blood pressure by oxidative stress and hyperglycemia

Because hydrogen peroxide is an obligate cosubstrate of MPO, we hypothesized that the impact of MPO on blood pressure would be most prominent on a background of oxidative stress. This was confirmed by our observation that the relationship between MPO and blood pressure was most prominent in individuals with a high plasma concentration of oxidized LDL. Our finding that high levels of fasting glucose strengthened the association between MPO and blood pressure is also compatible with this hypothesis, because high glucose concentrations have been shown to stimulate vascular production of reactive oxygen species.[10,24] HDL particles are known to have anti-inflammatory and antioxidative properties.[25] The observed decrease of the strength of the association between MPO and blood pressure with increasing levels of HDL-cholesterol is therefore also consistent with oxidative stress as a modifying factor. A high BMI, which is related to inflammation and oxidative stress,[26,27] and the metabolic syndrome, which captures much of the risk associated with the above-mentioned variables, were also found to enhance the relationship between MPO and blood pressure. Notably, all conditions that strengthened the association between MPO and blood pressure were associated with increased levels of oxidized LDL. All in all, the results of these analyses point in the same direction, ie, that the impact of MPO on blood pressure is most prominent in the presence of (glucose-induced) oxidative stress.

Potential confounding of the relationship between MPO and blood pressure by CRP

In contrast to the relationship between MPO and hypertension that has hardly been studied on the population level, in several studies an independent relationship between the inflammation marker CRP and hypertension was observed.[28-31] Notably, CRP has been reported to stimulate release of MPO from polymorphonuclear cells and monocytes.[32] This might indicate that the association between MPO and blood pressure is, at least partly, not causal but merely reflects the relationship between CRP and blood pressure. Indeed, in the present study MPO and CRP were

significantly correlated, albeit with moderate strength. However, in the linear regression models, the association between MPO and blood pressure was only slightly attenuated on adjustment for CRP, indicating that confounding of this association by CRP is of minor significance. Most likely, different mechanisms are involved in the associations of MPO and CRP with hypertension. This is consistent with CRP being mainly produced by the liver on stimulation by inflammatory cytokines, whereas MPO is locally produced at sites of inflammation including the vasculature.

Study limitations

First, although a causal relationship between MPO and blood pressure is plausible, we used a cross-sectional design, which does not allow us to draw definitive conclusions on causality. Second, although the original study population was recruited from the general population, for the present study a selection was made on the basis of glucose metabolism, ie, individuals with impaired glucose metabolism and type 2 diabetes were overrepresented. This design made this cohort very suitable to investigate the impact of (glucose-induced) oxidative stress on the relationship between MPO and hypertension. However, we cannot exclude the possibility that selection bias has influenced the relationship between MPO and blood pressure. Third, the study was performed in elderly white subjects, and the association between MPO and blood pressure may be different in younger individuals and other races. Fourth, MPO was measured in the circulation, and it is currently not known to what extent this measure reflects MPO in the vasculature. Finally, we measured MPO mass and not activity. However, a strong correlation between MPO mass and MPO activity (r=0.95) has been reported.[33]

Perspectives

The current study demonstrates that a high plasma level of MPO is associated with a clinically relevant increase in both SBP and DBP. The relationship between MPO and blood pressure is particularly prominent on a background of hyperglycemia or oxidative stress, consistent with the ability of MPO to amplify oxidative stress, eg, by using hydrogen peroxide as a cosubstrate to form more reactive oxidant species. The relationship between MPO and hypertension, together with emerging evidence

that MPO-derived oxidants contribute to initiation and propagation of acute and chronic vascular disease,[4-8] identifies MPO as an interesting target for drug development. However, because MPO plays an important role in the innate immune system, a suitable inhibitor should specifically target activity of MPO in the vascular wall, without interfering with its bactericidal activity.[34] Possibly, this selectivity may be achieved by lowering vascular oxidative stress and thereby the local concentration of hydrogen peroxide, the cosubstrate of MPO.

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Disclosures

None.

References

1. Li JJ, Fang CH, Hui RT. Is hypertension an inflammatory disease? *Med Hypotheses*. 2005;64:236-240.
2. Klebanoff SJ. Myeloperoxidase: friend and foe. *J Leukoc Biol*. 2005;77:598-625.
3. Nicholls SJ, Hazen SL. Myeloperoxidase and cardiovascular disease. *Arterioscler Thromb Vasc Biol*. 2005;25:1102-1111.
4. Brennan ML, Penn MS, Van Lente F, Nambi V, Shishehbor MH, Aviles RJ, Goormastic M, Pepoy ML, McErlean ES, Topol EJ, Nissen SE, Hazen SL. Prognostic value of myeloperoxidase in patients with chest pain. *N Engl J Med*. 2003;349:1595-1604.
5. Baldus S, Heeschen C, Meinertz T, Zeiher AM, Eiserich JP, Munzel T, Simoons ML, Hamm CW. Myeloperoxidase serum levels predict risk in patients with acute coronary syndromes. *Circulation*. 2003;108:1440-1445.
6. Mocatta TJ, Pilbrow AP, Cameron VA, Senthilmohan R, Frampton CM, Richards AM, Winterbourn CC. Plasma concentrations of myeloperoxidase predict mortality after myocardial infarction. *J Am Coll Cardiol*. 2007;49:1993-2000.
7. Meuwese MC, Stroes ES, Hazen SL, Van Miert JN, Kuivenhoven JA, Schaub RG, Wareham NJ, Luben R, Kastelein JJ, Khaw KT, Boekholdt SM. Serum myeloperoxidase levels are associated with the future risk of coronary artery disease in apparently healthy individuals: the EPIC-Norfolk Prospective Population Study. *J Am Coll Cardiol*. 2007;50:159-165.
8. Ali Z, Sarcia P, Mosley TH, Jr., Kondragunta V, Kullo IJ. Association of serum myeloperoxidase with the ankle-brachial index and peripheral arterial disease. *Vasc Med*. 2009;14:215-220.
9. Schindhelm RK, Van der Zwan LP, Teerlink T, Scheffer PG. Myeloperoxidase: a useful biomarker for cardiovascular disease risk stratification? *Clin Chem*. 2009;55:1462-1470.
10. Zhang C, Yang J, Jennings LK. Leukocyte-derived myeloperoxidase amplifies high-glucose--induced endothelial dysfunction through interaction with high-glucose--stimulated, vascular non--leukocyte-derived reactive oxygen species. *Diabetes*. 2004;53:2950-2959.
11. Mooy JM, Grootenhuis PA, De Vries H, Valkenburg HA, Bouter LM, Kostense PJ, Heine RJ. Prevalence and determinants of glucose intolerance in a Dutch caucasian population. The Hoorn Study. *Diabetes Care*. 1995;18:1270-1273.
12. Spijkerman AM, Adriaanse MC, Dekker JM, Nijpels G, Stehouwer CD, Bouter LM, Heine RJ. Diabetic patients detected by population-based stepwise screening already have a diabetic cardiovascular risk profile. *Diabetes Care*. 2002;25:1784-1789.
13. Alberti KG, Zimmet PZ. Definition, diagnosis and classification of diabetes mellitus and its complications. Part 1: diagnosis and classification of diabetes mellitus provisional report of a WHO consultation. *Diabet Med*. 1998;15:539-553.
14. Chobanian AV, Bakris GL, Black HR, Cushman WC, Green LA, Izzo JL, Jr., Jones DW, Materson BJ, Oparil S, Wright JT, Jr., Roccella EJ. Seventh report of the Joint National Committee on Prevention, Detection, Evaluation, and Treatment of High Blood Pressure. *Hypertension*. 2003;42:1206-1252.

15. Scheffer PG, Van der Zwan LP, Schindhelm RK, Vermue HP, Teerlink T. Myeloperoxidase concentrations in EDTA-plasma of healthy subjects are discordant with concentrations in heparin-plasma and serum. *Clin Biochem*. 2009;42:1490-1492.
16. Grooteman MP, Gritters M, Wauters IM, Schalkwijk CG, Stam F, Twisk J, Ter Wee PM, Nube MJ. Patient characteristics rather than the type of dialyser predict the variability of endothelial derived surface molecules in chronic haemodialysis patients. *Nephrol Dial Transplant*. 2005;20:2751-2758.
17. Van der Zwan LP, Teerlink T, Dekker JM, Henry RM, Stehouwer CD, Jakobs C, Heine RJ, Scheffer PG. Circulating oxidized LDL: determinants and association with brachial flow-mediated dilation. *J Lipid Res*. 2009;50:342-349.
18. Expert Panel on Detection, Evaluation, And Treatment of High Blood Cholesterol In Adults (Adult Treatment Panel III). Executive Summary of The Third Report of The National Cholesterol Education Program (NCEP). *J Am Med Assoc*. 2001;285:2486-2497.
19. Prineas RJ, Crow RS, Blackburn H. *The Minnesota Code Manual for Electrocardiographic Findings: Standards and Procedures for Measurement and Classification*. Boston, Mass: J. Wright, 1982.
20. Abu-Soud HM, Hazen SL. Nitric oxide is a physiological substrate for mammalian peroxidases. *J Biol Chem*. 2000;275:37524-37532.
21. Baldus S, Heitzer T, Eiserich JP, Lau D, Mollnau H, Ortak M, Petri S, Goldmann B, Duchstein HJ, Berger J, Helmchen U, Freeman BA, Meinertz T, Munzel T. Myeloperoxidase enhances nitric oxide catabolism during myocardial ischemia and reperfusion. *Free Radic Biol Med*. 2004;37:902-911.
22. Yang J, Ji R, Cheng Y, Sun JZ, Jennings LK, Zhang C. L-arginine chlorination results in the formation of a nonselective nitric-oxide synthase inhibitor. *J Pharmacol Exp Ther*. 2006;318:1044-1049.
23. Xu J, Xie Z, Reece R, Pimental D, Zou MH. Uncoupling of endothelial nitric oxidase synthase by hypochlorous acid: role of NAD(P)H oxidase-derived superoxide and peroxynitrite. *Arterioscler Thromb Vasc Biol*. 2006;26:2688-2695.
24. Guzik TJ, Mussa S, Gastaldi D, Sadowski J, Ratnatunga C, Pillai R, Channon KM. Mechanisms of increased vascular superoxide production in human diabetes mellitus: role of NAD(P)H oxidase and endothelial nitric oxide synthase. *Circulation*. 2002;105:1656-1662.
25. Kontush A, Chapman MJ. Functionally defective high-density lipoprotein: a new therapeutic target at the crossroads of dyslipidemia, inflammation, and atherosclerosis. *Pharmacol Rev*. 2006;58:342-374.
26. Libby P. Inflammation in atherosclerosis. *Nature*. 2002;420:868-874.
27. Keaney JF, Jr., Larson MG, Vasan RS, Wilson PW, Lipinska I, Corey D, Massaro JM, Sutherland P, Vita JA, Benjamin EJ. Obesity and systemic oxidative stress: clinical correlates of oxidative stress in the Framingham Study. *Arterioscler Thromb Vasc Biol*. 2003;23:434-439.
28. King DE, Egan BM, Mainous AG, III, Geesey ME. Elevation of C-reactive protein in people with prehypertension. *J Clin Hypertens (Greenwich)*. 2004;6:562-568.

29. Lakoski SG, Cushman M, Palmas W, Blumenthal R, D'Agostino RB, Jr., Herrington DM. The relationship between blood pressure and C-reactive protein in the Multi-Ethnic Study of Atherosclerosis (MESA). *J Am Coll Cardiol*. 2005;46:1869-1874.
30. Sesso HD, Buring JE, Rifai N, Blake GJ, Gaziano JM, Ridker PM. C-reactive protein and the risk of developing hypertension. *J Am Med Assoc*. 2003;290:2945-2951.
31. Bautista LE, Lopez-Jaramillo P, Vera LM, Casas JP, Otero AP, Guaracao AI. Is C-reactive protein an independent risk factor for essential hypertension? *J Hypertens*. 2001;19:857-861.
32. Singh U, Devaraj S, Jialal I. C-reactive protein stimulates myeloperoxidase release from polymorphonuclear cells and monocytes: implications for acute coronary syndromes. *Clin Chem*. 2009;55:361-364.
33. Zhang R, Brennan ML, Fu X, Aviles RJ, Pearce GL, Penn MS, Topol EJ, Sprecher DL, Hazen SL. Association between myeloperoxidase levels and risk of coronary artery disease. *J Am Med Assoc*. 2001;286:2136-2142.
34. Malle E, Furtmuller PG, Sattler W, Obinger C. Myeloperoxidase: a target for new drug development? *Br J Pharmacol*. 2007;152:838-854.

Chapter 6B

Reduction of myeloperoxidase activity by melatonin and pycnogenol may contribute to their blood pressure lowering effect

Leonard P. van der Zwan, Peter G. Scheffer and Tom Teerlink

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To the Editor:

In a recent issue of *Hypertension*, Rezzani et al reported that 6 weeks of treatment with either melatonin or pycnogenol, a pine bark extract rich in flavonoids, protected structure and function of the microvasculature in spontaneously hypertensive rats and resulted in a reduction in systolic blood pressure.[1] These effects were ascribed to the antioxidant properties of both compounds that reduce oxidative stress and increase the availability of nitric oxide by several mechanisms as depicted in the figure in the accompanying editorial.[2] We would like to propose reduction of the activity of the oxidative enzyme myeloperoxidase (MPO) as an additional mechanism.

MPO, an enzyme linked to both inflammation and oxidative stress, catalyzes the production of hypochlorous acid and a range of other highly reactive species, which, by killing pathogens, play a protective role in the innate immune response. In the vasculature, however, these MPO-derived reactive substances may lead to structural damage and reduce the bioavailability of the endogenous vasodilator nitric oxide. Accordingly, MPO is associated with the initiation and propagation of cardiovascular disease.[3] We recently observed a positive association between levels of MPO and both systolic and diastolic blood pressure in a population-based cohort of elderly subjects.[4] Because hydrogen peroxide is an obligate cosubstrate of MPO, the activity of MPO in the vasculature is enhanced by increased local production of reactive oxygen species. In other words, MPO has the ability to amplify oxidative stress, by using hydrogen peroxide to form reactive oxidant species with a higher oxidative potential. In accordance with this notion, the relationship between MPO and blood pressure was strongest in individuals with features associated with increased oxidative stress, such as obesity, low levels of high-density lipoprotein cholesterol, the metabolic syndrome, and type 2 diabetes.[4]

Interestingly, Galijasevic et al identified melatonin as a potent inhibitor of MPO.[5] They showed that, at physiological and supplemental concentrations, melatonin interferes with the catalytic activity of MPO by multiple pathways, including switching the activity of MPO from peroxidation to catalase-like activity and conversion of MPO to an inactive form.

Next to inhibition of MPO, melatonin may also reduce the activity of MPO in the vasculature by 2 other mechanisms that may also apply to pycnogenol. First, both

compounds are potent scavengers of reactive oxygen species and may thereby limit the production of hydrogen peroxide, the cosubstrate of MPO. Second, the anti-inflammatory properties of melatonin and pycnogenol may reduce infiltration of the vascular wall by MPO-secreting leukocytes.

In summary, we propose that, in addition to the mechanisms suggested by Rezzani et al, a reduction of vascular MPO activity likely contributes to the vasoprotective and blood pressure-lowering effects of melatonin and pycnogenol.

Disclosures

None.

References

1. Rezzani R, Porteri E, De Ciuceis C, Bonomini F, Rodella LF, Paiardi S, Boari GE, Platto C, Pilu A, Avanzi D, Rizzoni D, Agabiti RE. Effects of melatonin and Pycnogenol on small artery structure and function in spontaneously hypertensive rats. *Hypertension*. 2010;55:1373-1380.
2. Ferri C, Grassi D. Antioxidants and beneficial microvascular effects: is this the remedy? *Hypertension*. 2010;55:1310-1311.
3. Schindhelm RK, Van der Zwan LP, Teerlink T, Scheffer PG. Myeloperoxidase: a useful biomarker for cardiovascular disease risk stratification? *Clin Chem*. 2009;55:1462-1470.
4. Van der Zwan LP, Scheffer PG, Dekker JM, Stehouwer CD, Heine RJ, Teerlink T. Hyperglycemia and oxidative stress strengthen the association between myeloperoxidase and blood pressure. *Hypertension*. 2010;55:1366-1372.
5. Galijasevic S, Abdulhamid I, Abu-Soud HM. Melatonin is a potent inhibitor for myeloperoxidase. *Biochemistry*. 2008;47:2668-2677.

Chapter 7

Systemic inflammation is linked to low arginine and high ADMA plasma levels resulting in an unfavorable NOS substrate-to-inhibitor ratio – the Hoorn Study

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Submitted for publication

Abstract

Objective: Inflammation is associated with a reduced availability of nitric oxide (NO) in the vasculature. We investigated the possible involvement of altered levels of substrate (arginine) and inhibitor (asymmetric dimethylarginine; ADMA) of NO synthase (NOS).

Methods: Plasma concentrations of arginine and ADMA, the inflammatory markers C-reactive protein (CRP) and myeloperoxidase (MPO), and oxidized LDL (oxLDL) were measured in 369 male and 377 female participants (aged 50 to 87 years) of a population-based cohort study.

Results: The arginine/ADMA ratio decreased significantly across increasing tertiles of CRP and MPO. These negative associations remained significant in a linear regression model with both MPO (P=0.002) and CRP (P<0.001) as independent variables and adjusted for age, sex and cardiovascular risk factors. In a fully adjusted regression model, MPO was positively associated with ADMA (5.4 [95% CI 1.3 to 9.4] nmol/L change of ADMA per SD increase of MPO; P=0.010), whereas CRP was not (P=0.36). Conversely, in a fully adjusted model, CRP was negatively associated with arginine (-2.8 [95% CI -4.0 to -1.6] µmol/L arginine per SD of CRP; P<0.001), without a significant contribution of MPO (P=0.23). The relationship between MPO and ADMA became stronger with increasing levels of oxLDL (1.8, 5.2, and 8.7 nmol/L ADMA per SD of MPO for increasing tertiles of oxLDL), consistent with the ability of MPO to amplify oxidative stress. In contrast, the relationship between CRP and arginine was not modified by levels of oxLDL.

Conclusion: An unfavorable NOS substrate/inhibitor ratio may contribute to the reduced NO bioavailability associated with inflammation.

Introduction

Endothelium-derived nitric oxide (NO), which is synthesized from arginine by NO synthase (NOS), is an important regulator of vascular homeostasis. In addition to being a powerful endogenous vasodilator, NO inhibits the adhesion of inflammatory cells to the vascular wall, the aggregation of platelets, and the proliferation of smooth muscle cells.[1] Inactivation and/or reduced synthesis of NO is seen in conjunction with risk factors for cardiovascular disease (CVD) and may promote endothelial dysfunction, hypertension, thrombus formation, and atherogenesis.[1-3]

Infusion or chronic oral administration of arginine has been shown to increase NO production, suggesting that under physiological conditions substrate availability affects NO production.[4] NO production can be inhibited by the endogenous NOS inhibitor asymmetric dimethylarginine (ADMA), which originates from the proteolysis of methylated proteins.[5] Increased plasma levels of ADMA have been found to predict future CVD events, independent of traditional risk factors.[6,7] Therefore, on top of other regulatory mechanisms, NO production is determined by both substrate availability (arginine) and the presence of inhibitor (ADMA), which may be adequately reflected by their ratio.[8]

Inflammation is involved in all phases of the atherosclerotic process.[9] The inflammatory markers C-reactive protein (CRP) and myeloperoxidase (MPO) are both associated with endothelial dysfunction,[10-12] possibly due to a reduced bioavailability of NO, but whether low-grade inflammation is associated with altered levels of arginine and/or ADMA has not been thoroughly investigated. We have addressed this issue by studying how concentrations of arginine and ADMA are related to concentrations of CRP and MPO in plasma samples from 746 participants of a population-based cohort study, the Hoorn Study.

Methods

Subjects

For the present investigation we used data from the 2000 Hoorn Study follow-up examination and the Hoorn Screening Study, which are population-based studies on glucose metabolism and cardiovascular disease in a white population. This study population has been described in detail elsewhere.[13] From the 822 participants, we excluded 74 subjects with missing data on primary variables of interest and two subjects with extreme values for arginine or ADMA. In total 746 subjects (369 men and 377 women) remained, of whom 267 had a normal glucose metabolism, 190 had impaired glucose metabolism and 289 had type 2 diabetes according to WHO 1999 criteria.[14] The local ethics committee approved the study, and all participants gave their written informed consent.

Biochemical analyses

Plasma concentrations of arginine, ADMA, and symmetric dimethylarginine (SDMA) were measured by high-performance liquid chromatography (HPLC) with fluorescence detection as earlier described,[15] using modified chromatographic separation conditions.[16] The intra-assay and inter-assay coefficients of variation (CV) for all analytes were <1.5% and <4.0%, respectively. Plasma CRP concentrations were determined with a highly sensitive in-house sandwich enzyme-linked immunosorbent assay (ELISA), with an intra-assay CV of 3.9% and inter-assay CV of 8.7%.[17] A sandwich ELISA (Mercodia, Uppsala, Sweden) was used to determine MPO concentrations in EDTA-plasma, with intra- and inter-assay CVs of 3.9% and 5.0%, respectively.[18] Apolipoprotein-B100 (apoB100) concentrations were determined nephelometrically using an “Immage 800” immunochemistry system (Beckman Coulter, Fullerton, CA). Circulating plasma oxidized LDL (oxLDL) was determined by competitive ELISA (Mercodia) with intra- and inter-assay CVs of 6.7% and 7.0%, respectively.[19] HbA1c was analyzed by ion-exchange HPLC on a modular diabetes monitoring system (Bio-Rad, Veenendaal, The Netherlands). Glucose was measured enzymatically (Roche, Mannheim, Germany) and insulin was determined by a two-site immuno-radiometric assay (Medgenix, Diagnostics, Fleurus, Belgium). Total cholesterol, HDL-cholesterol and triglycerides (TG) were measured by standard enzymatic methods (Roche). LDL-cholesterol concentration was determined with a

direct method with the “N-geneous” assay (GenZyme, Cambridge, MA, USA). With this method, a triglyceride concentration up to 13.5 mmol/L does not interfere with measurement of LDL-cholesterol.

Renal function and cardiovascular disease

Microalbuminuria was defined as a urinary albumin/creatinine ratio ≥2.0 mg/mmol. Glomerular filtration rate was estimated using the 4-variable Modification of Diet in Renal Disease (MDRD) Study equation.[20] Prior cardiovascular disease was defined as a history of angina, claudication, myocardial infarction, transient ischemic attack or ischemic stroke, abnormalities on a resting ECG (Minnesota codes 1.1-1.3, 4.1-4.3, 5.1-5.3 or 7.1), undergone coronary bypass surgery or angioplasty, peripheral arterial bypass or non-traumatic amputation and/or ankle-brachial index of <0.9 in either leg.

Statistics

Unless otherwise indicated, data are presented as mean and SD or, if skewed, median and interquartile range. Skewed variables, notably CRP and MPO, were natural log-transformed before statistical analyses. Student’s t-test was applied for comparison of variables between two groups. Linear regression analysis was used to assess the relationships of CRP and MPO with arginine, ADMA, and their ratio. To adjust for mutual confounding, both CRP and MPO were entered together as independent variables in multivariable regression models with arginine, ADMA or the arginine/ADMA ratio as dependent variable. Subsequently these models were further adjusted for age and sex alone, or additionally for glucose tolerance status and the following cardiovascular risk factors: waist circumference, LDL- and HDL-cholesterol, current smoking, and estimated glomerular filtration rate. Regression coefficients were expressed as change in the dependent variable per SD increase of CRP or MPO. Data were analyzed using SPSS software, version 17 (SPSS Inc., Chicago, IL). A two-tailed probability value <0.05 was considered to indicate statistical significance.

Results

Increased levels of CRP and MPO are associated with a decreased arginine/ADMA ratio

Subject characteristics are shown in Table 1.

Table 1. Subject characteristics

Variable	Unit	
N		746
Age	years	68.8 (7.2)
Sex, male	%	49
Arginine	µmol/L	94.1 (15.6)
ADMA	µmol/L	0.449 (0.058)
Arginine/ADMA ratio	µmol/µmol	212.1 (38.5)
SDMA	µmol/L	0.498 (0.110)
Myeloperoxidase	µg/L	56.1 (47.4-67.0)
C-reactive protein	mg/L	2.20 (1.06-4.68)
HbA1c	%	6.1 (0.8)
Fasting glucose	mmol/L	6.1 (5.5-7.0)
Insulin	pmol/L	62 (43-90)
LDL-cholesterol	mmol/L	3.6 (0.9)
HDL-cholesterol	mmol/L	1.38 (0.40)
Triglycerides	mmol/L	1.4 (1.0-1.9)
Apolipoprotein-B100	g/L	1.02 (0.23)
Oxidized LDL	U/L	64.8 (15.2)
OxLDL/apoB100 ratio	U/g	64.0 (9.7)
Lipid lowering medication	%	18
Body mass index	kg/m^2	27.7 (4.2)
Waist circumference	cm	96.5 (12.2)
Systolic blood pressure	mm Hg	142 (20)
Diastolic blood pressure	mm Hg	83 (11)
Antihypertensive medication	%	39
Current smoking	%	15
(Micro-)albuminuria	%	15
Glomerular filtration rate	ml/min per 1.73 m^2	60.5 (10.6)
Prior cardiovascular disease	%	56

Mean (standard deviation) or for skewed variables median (interquartile range) are shown. ADMA, asymmetric dimethylarginine; SDMA, symmetric dimethylarginine.

Plasma concentrations of CRP, MPO, and arginine did not differ between men and women, but women had significantly higher ADMA levels (0.459 ± 0.053 versus 0.438 ± 0.060 µmol/L; *P*<0.001) and a lower arginine/ADMA ratio (204.3 ± 33.2 versus 220.0 ± 41.7; *P*<0.001) than men. The arginine/ADMA ratio decreased across increasing tertiles of CRP and MPO (Figure 1A and B) and these trends were highly significant after adjustment for age and sex (*P*<0.001 for both CRP and MPO).

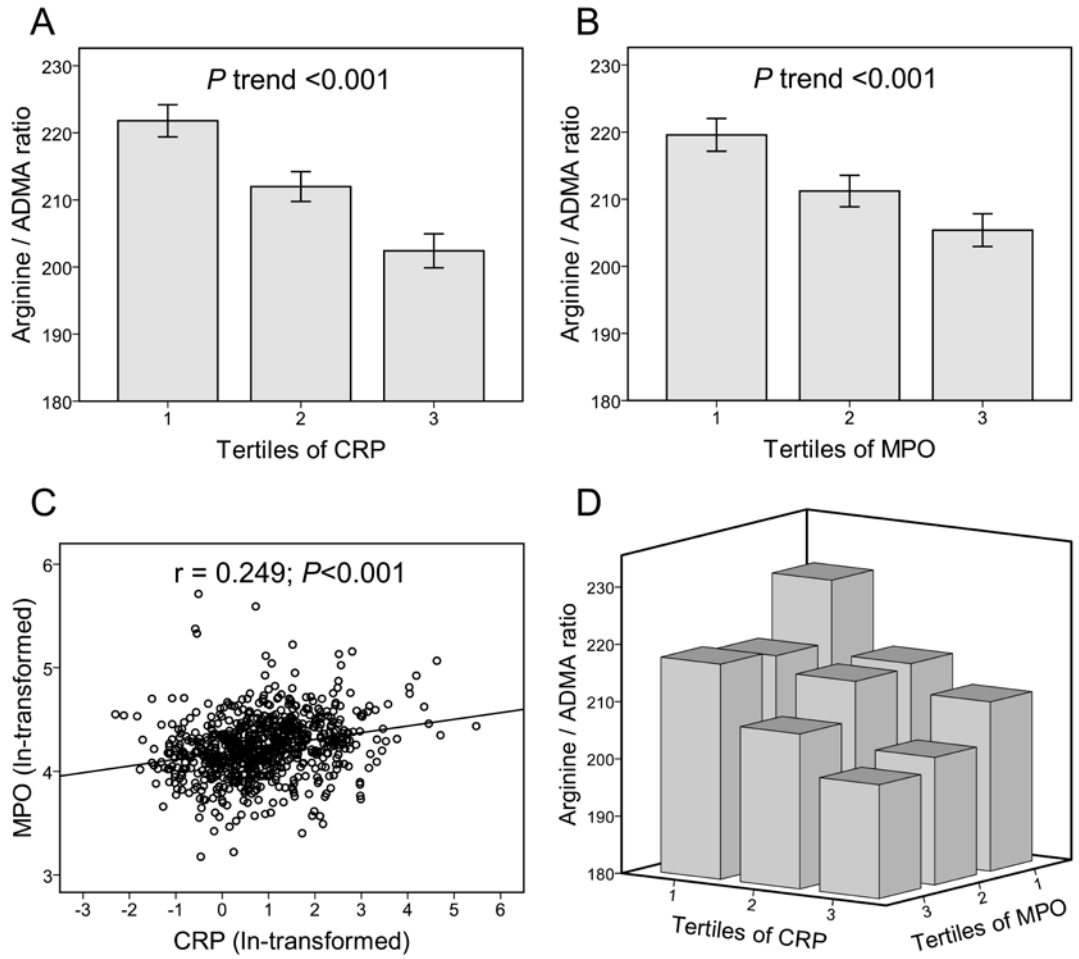

Figure 1. Mean (SEM) arginine/ADMA ratio in plasma of subjects stratified by tertiles of CRP (A), or tertiles of MPO (B), the relation between plasma levels of CRP and MPO (C), and the mean Arginine/ADMA ratio after two-dimensional stratification of the cohort by tertiles of CRP and MPO (D). P for trend values (A and B) were derived from age- and sex-adjusted linear regression analyses and the strength of the association (C) as indicated by Pearson's correlation coefficient was calculated after logarithmic transformation.

Although CRP and MPO were significantly correlated (Figure 1C), this relation was not very strong, indicating that both inflammatory markers, at least to some extent, also carry different information. In line with this, two-dimensional stratification of the cohort by tertiles of CRP and MPO revealed that in each tertile of CRP, increasing levels of MPO were associated with a decrease of the arginine/ADMA ratio. Likewise, in each stratum of MPO the arginine/ADMA ratio decreased with increasing levels of CRP (Figure 1D). In subjects with both CRP and MPO in the highest tertile the mean arginine/ADMA ratio was 200, 12% lower than the mean ratio of 227 in subjects with both CRP and MPO in the lowest tertile, the difference being equivalent to ~0.7 times the standard deviation of the arginine/ADMA ratio in the entire cohort.

Independent associations between CRP and arginine and between MPO and ADMA

To investigate whether the associations of both inflammatory markers with the arginine/ADMA ratio were independent of age, sex, and established cardiovascular risk factors we explored linear regression models (Table 2). In separate regression models, both CRP and MPO were significantly associated with the arginine/ADMA ratio and when mutual adjustment was performed by entering both CRP and MPO in the same regression model, these associations were slightly attenuated but still highly significant, also after further adjustment for age, sex, glucose tolerance status, and established cardiovascular risk factors.

Next, we separately explored the associations of CRP and MPO with enumerator and denominator of the arginine/ADMA ratio by linear regression analyses (Table 2). In crude models, both CRP and MPO showed a significant negative association with arginine and a significant positive association with ADMA. Upon mutual adjustment, however, only CRP remained significantly associated with arginine, whereas the relation between MPO and arginine lost significance. Conversely, in the mutually adjusted model only MPO retained its significant association with ADMA, whereas the relation between CRP and ADMA lost significance. The negative association between CRP and arginine and the positive association between MPO and ADMA were only slightly attenuated and remained highly significant after further adjustment for age, sex, glucose tolerance status, and established cardiovascular risk factors. To investigate whether these associations

were subject to modification by glucose metabolism, regression analyses were repeated after stratification for glucose tolerance status. The age- and sex-adjusted regression coefficients of the relation between CRP and arginine and the relation between MPO and ADMA did not differ significantly between subjects with normal glucose metabolism, impaired glucose metabolism, and type 2 diabetes (P>0.2 for all comparisons).

We also investigated potential relationships of CRP and MPO with SDMA. In unadjusted regression models, CRP was positively associated with SDMA (10.1 [95% CI 2.2 to 18.0] nmol/L SDMA per standard deviation increase of MPO; P=0.012), whereas MPO was not (P=0.19). In a mutually and age- and sex-adjusted model neither CRP (P=0.17) nor MPO (P=0.88) were significantly related to SDMA.

Table 2. Linear regression models for the relations of CRP and MPO with arginine, ADMA, and their ratio

Model		Arginine / ADMA ratio		Arginine		ADMA	
		Beta (95% CI)	*P*-value	Beta (95% CI)	*P*-value	Beta (95% CI)	*P*-value
1. Crude	CRP	-9.9 (-12.5 to -7.2)	<0.001	-3.2 (-4.3 to -2.2)	<0.001	4.9 (0.8 to 9.0)	0.020
	MPO	-7.0 (-9.7 to -4.3)	<0.001	-1.4 (-2.5 to -0.2)	0.017	7.5 (3.4 to 11.6)	<0.001
2. Mutually adjusted	CRP	-8.7 (-11.4 to -5.9)	<0.001	-3.1 (-4.2 to -2.0)	<0.001	3.2 (-1.0 to 7.5)	0.13
	MPO	-4.8 (-7.6 to -2.1)	<0.001	-0.6 (-1.7 to 0.5)	0.31	6.7 (2.4 to 10.9)	0.002
3. Mutually and age- and sex-adjusted	CRP	-8.5 (-11.1 to -5.8)	<0.001	-3.2 (-4.3 to -2.0)	<0.001	2.3 (-1.7 to 6.4)	0.26
	MPO	-4.1 (-6.7 to -1.4)	0.003	-0.6 (-1.7 to 0.5)	0.31	5.1 (1.0 to 9.2)	0.014
4. 3 + glucose tolerance status	CRP	-8.5 (-11.2 to -5.8)	<0.001	-3.0 (-4.2 to -1.9)	<0.001	3.2 (-1.0 to 7.3)	0.13
	MPO	-4.0 (-6.7 to -1.4)	0.003	-0.5 (-1.7 to 0.6)	0.34	5.3 (1.2 to 9.3)	0.011
5. 3 + waist circumference	CRP	-7.5 (-10.3 to -4.7)	<0.001	-2.7 (-3.9 to -1.5)	<0.001	2.8 (-1.6 to 7.1)	0.21
	MPO	-4.0 (-6.7 to -1.3)	0.004	-0.6 (-1.7 to 0.6)	0.34	5.1 (1.0 to 9.3)	0.015
6. 3 + LDL- and HDL-cholesterol	CRP	-7.8 (-10.5 to -5.0)	<0.001	-3.0 (-4.2 to -1.8)	<0.001	1.7 (-2.6 to 5.9)	0.44
	MPO	-4.2 (-6.8 to -1.5)	0.002	-0.6 (-1.8 to 0.5)	0.29	5.2 (1.1 to 9.3)	0.013
7. 3 + current smoking	CRP	-8.8 (-11.4 to -6.1)	<0.001	-3.4 (-4.5 to -2.3)	<0.001	1.8 (-2.3 to 5.9)	0.39
	MPO	-4.3 (-7.0 to -1.6)	0.002	-0.8 (-1.9 to 0.4)	0.19	4.7 (0.6 to 8.8)	0.023
8. 3 + glomerular filtration rate	CRP	-8.5 (-11.1 to -5.8)	<0.001	-3.2 (-4.3 to -2.1)	<0.001	2.3 (-1.8 to 6.3)	0.27
	MPO	-4.1 (-6.8 to -1.4)	0.003	-0.6 (-1.7 to 0.6)	0.32	5.2 (1.1 to 9.2)	0.012
9. Fully adjusted*	CRP	-7.5 (-10.4 to -4.6)	<0.001	-2.8 (-4.0 to -1.6)	<0.001	2.0 (-2.3 to 6.3)	0.36
	MPO	-4.4 (-7.1 to -1.7)	0.002	-0.7 (-1.8 to 0.4)	0.23	5.4 (1.3 to 9.4)	0.010

Beta values represent increase in the arginine/ADMA ratio, arginine concentration (µmol/L) or ADMA concentration (nmol/L) per 1-SD increment of CRP or MPO. *Mutually adjusted model, additionally adjusted for age, sex, glucose tolerance status, LDL- and HDL-cholesterol, smoking, and glomerular filtration rate.

Oxidative stress strengthens the relation between MPO and ADMA

Because hydrogen peroxide is an obligate cosubstrate of MPO, the activity of MPO is enhanced by increased production of reactive oxygen species. To test whether the relation between MPO and ADMA was strengthened under conditions of oxidative stress, we repeated the regression analyses after stratification of the cohort according to plasma levels of oxidized LDL. In age- and sex-adjusted regression models in which both MPO and CRP were used as independent variables, the strength of the association between MPO and ADMA was stronger at higher levels of oxLDL (1.8, 5.2, and 8.7 nmol/L ADMA per standard deviation of MPO for increasing tertiles of oxLDL; Figure 2A). In contrast, the strength of the association between plasma levels of CRP and arginine did not differ between strata of oxLDL (Figure 2B).

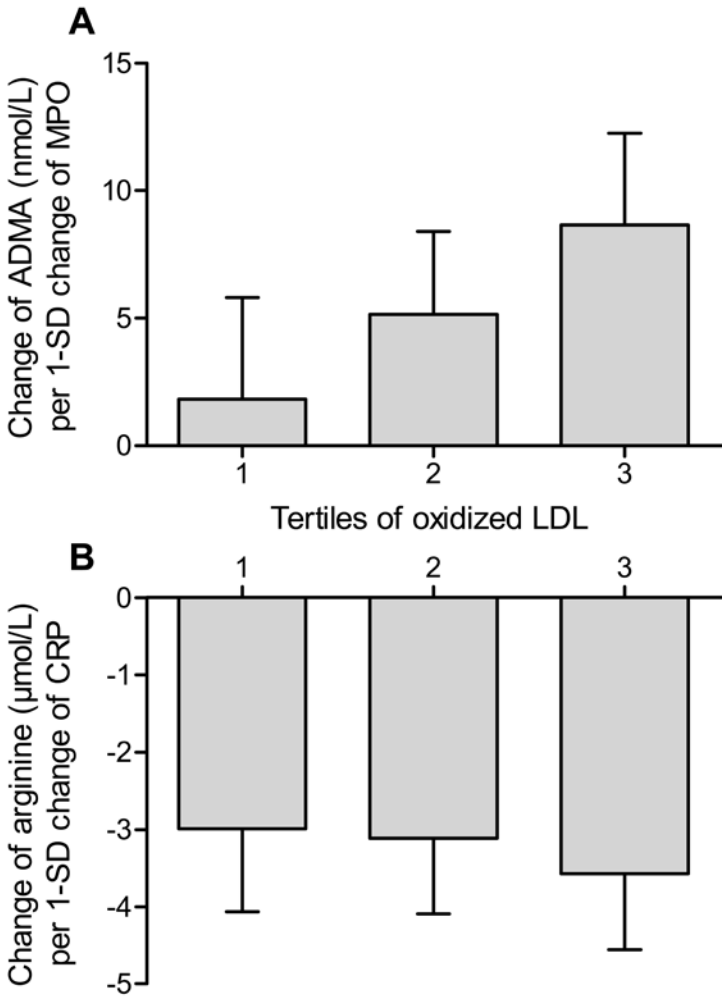

Figure 2. Influence of oxidative stress, assessed by stratification based on plasma levels of oxidized LDL, on the relationship between plasma levels of MPO and ADMA (A) and the relationship between plasma levels of CRP and arginine (B). The strengths of the relations was derived from mutually-, age- and sex-adjusted linear regression models and expressed as change in concentration of the dependent variable (ADMA or arginine) per 1 standard deviation of the independent variable (MPO or CRP). MPO and CRP were natural log-transformed prior to analysis. Error bars represent SE.

It should be noted that the amount of oxLDL in the circulation not only depends on oxidative stress but also on the total amount of LDL particles. By using the ratio of oxLDL over apoB100, adjustment for particle number is made and, therefore, this ratio may more accurately reflect oxidative stress. The increase of the strength of the association between MPO and ADMA across tertiles of the oxLDL/apoB100 ratio (1.4, 4.4, and 11.1 nmol/L ADMA per standard deviation of MPO for increasing tertiles of the oxLDL/apoB100 ratio) was slightly more pronounced as compared to the increasing trend across tertiles of oxLDL.

Discussion

The most salient finding of this study is that low-grade systemic inflammation was associated with a decreased arginine/ADMA ratio, reflecting a low NOS substrate/inhibitor ratio. Two striking differences between the inflammatory markers CRP and MPO were apparent. First, elevated levels of both markers were associated with a low arginine/ADMA ratio, but in the case of CRP this was mainly caused by a negative association with arginine, and in the case of MPO by a positive association with ADMA. Second, oxLDL strengthened the association between MPO and ADMA, consistent with the ability of MPO to amplify oxidative stress, whereas the association between CRP and arginine was not modified by levels of oxLDL.

Arginine is one of the most versatile amino acids, serving as a building block in protein synthesis and as a precursor for the synthesis of NO, urea, polyamines, proline, glutamate, creatine and agmatine.[21] The biochemical pathways involved share, and under some circumstances compete for, the available amounts of arginine. Endothelial cells for example express arginases that compete with NOS for substrate and, if highly expressed, may “starve” endothelial NOS.[3] Evidence for a role of increased arginase activity in endothelial dysfunction has been provided in animal models of CVD,[3] and endothelial arginase has been identified as a target for treatment of atherosclerosis.[22] Inflammation-induced upregulation of arginase activity seems a plausible explanation for the inverse association between plasma levels of CRP and arginine observed in this study. A similar negative association between CRP and arginine has also been reported in critically ill children.[23]

Both ADMA and SDMA are generated by methylation of arginine residues in proteins and released in the cytosol as free amino acids during proteolysis. ADMA is

largely cleared through intracellular metabolism by dimethylarginine dimethylaminohydrolase (DDAH), whereas SDMA is mainly cleared by renal excretion.[5,24] Theoretically, plasma levels of ADMA can rise by increased generation, decreased clearance, or altered distribution between the circulation and intracellular stores. Although the design of our study does not allow discriminating between these possibilities, the results provide some pointers to the mechanisms most likely involved. The fact that MPO was positively related to ADMA, but not to SDMA, argues against involvement of mechanisms shared by both methylarginines, such as protein methylation and proteolysis. Also a change in glomerular filtration rate is not a likely cause, because this would affect SDMA more than ADMA.[25] This leaves a change in the activity of DDAH, that degrades ADMA but nor SDMA, as a likely candidate. DDAH, which has a critical sulfhydryl group in its catalytic site, is sensitive to oxidative stress that may reversibly decrease its activity.[24,26] The fact that MPO, but not CRP, was associated with ADMA is compatible with oxidative stress-induced inactivation of DDAH, because only MPO is directly linked to oxidative stress.[27] Because hydrogen peroxide is an obligate cosubstrate of MPO, the activity of MPO in the vasculature may be enhanced by increased local production of reactive oxygen species.[27,28] Indeed, we observed that the relation between MPO and ADMA was strongest in individuals with a high plasma level of oxLDL. The impact of oxidative stress on the relation between MPO and ADMA was slightly more prominent if stratification was performed on the basis of the oxLDL/apoB100 ratio, consistent with our previous finding that this ratio may better reflect oxidative stress.[19]

Figure 3 schematically depicts our view that the inflammation-associated decreased levels of arginine and increased levels of ADMA are caused by upregulation of arginase and inhibition of DDAH activity, respectively. Both pathways result in lowering of the NOS substrate/inhibitor ratio, leading to a diminished NO production and endothelial dysfunction. The existence of these separate pathways is consistent with CRP being mainly produced by the liver, whereas MPO is locally produced at sites of inflammation including the vasculature. It should be noted, however, that there may be extensive crosstalk between both pathways. CRP has for instance been shown to stimulate MPO release from polymorphonuclear cells and monocytes,[29] which may in part explain the association between both markers observed in the present study. Furthermore, both CRP and MPO may negatively

affect the bioavailability of NO in ways that are independent of the arginine/ADMA ratio, such as direct scavenging of NO and NOS uncoupling.[27,30]

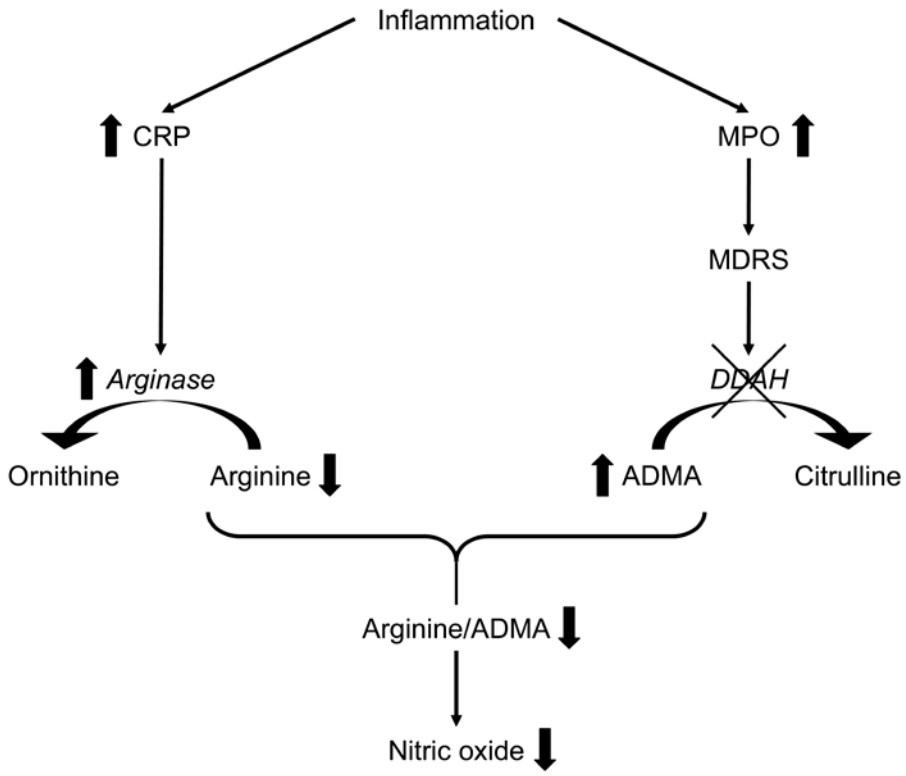

Figure 3. Potential causal pathways by which inflammation and associated oxidative stress can reduce the NOS substrate/inhibitor ratio in the vasculature. Low-grade inflammation, as reflected by increased CRP levels, is associated with increased expression of the inducible isoform of arginase, resulting in lower arginine levels. Inflammation is also associated with release of MPO in the vascular wall, where MPO-derived reactive substances (MDRS) may oxidatively inactivate the ADMA-degrading enzyme dimethylarginine dimethylaminohydrolase (DDAH), resulting in increased ADMA levels. Both routes contribute to a lowering of the arginine/ADMA ratio resulting in reduced nitric oxide output.

The present study has some limitations that deserve mention. First, arginine and ADMA were measured in the circulation, but both NO synthesis and ADMA generation are intracellular events. It is therefore not clear whether the circulating levels of arginine and ADMA appropriately reflect their levels at the site of NOS. On a parallel note, it is not clear to what extent circulatory levels of MPO, CRP, and oxLDL reflect local levels in the vasculature. However, the circulating level of ADMA has

been shown to predict future cardiovascular events in several prospective cohort studies,[6,7] and inverse associations of circulatory levels of CRP, MPO, and oxLDL with vascular function have been demonstrated,[10-12,19] suggesting a biological relevance of circulating levels. Second, the present study was conducted in white elderly subjects and therefore the results may be different in other ethnic and age groups. Third, the cross-sectional design of the study limits the possibility to draw definitive conclusions on causality. Major strengths of our study include the large number of subjects and the availability of a wide array of clinical and biochemical variables to control for potential confounding.

Conclusion

Endothelial production of adequate amounts of NO is essential for maintaining proper vascular function. Shortage of NO leads to endothelial dysfunction and accelerates atherogenesis, whereas an excess of NO may cause circulatory shock. The importance of control of NO availability is underlined by the fact that NOS activity is regulated at many levels, including transcription, translation, phosphorylation and other posttranslational modifications, interactions with other proteins and the plasma membrane, binding of cofactors, and the local concentrations of substrate (arginine) and endogenous inhibitor (ADMA). Although the reduced availability of NO that is associated with systemic inflammation most likely cannot be ascribed to a single regulatory level, the results of the present study show that a decrease of arginine and an increase of ADMA concentrations may be contributing factors. In addition, oxidative stress seems to enhance the association between inflammation and increased ADMA levels. Taken together, these findings suggest that improvement of NO production by an increase of the substrate/inhibitor ratio may be best achieved by combined management of inflammation and oxidative stress.

Conflict of interest statement

The authors have no conflicts of interest

Acknowledgements

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References

1. Moncada S, Higgs EA. The discovery of nitric oxide and its role in vascular biology. *Br J Pharmacol*. 2006;147:S193-S201.
2. Vanhoutte PM, Shimokawa H, Tang EH, Feletou M. Endothelial dysfunction and vascular disease. *Acta Physiol (Oxf)*. 2009;196:193-222.
3. Förstermann U. Nitric oxide and oxidative stress in vascular disease. *Pflugers Arch*. 2010;459:923-939.
4. Tsikas D, Böger RH, Sandmann J, Bode-Böger SM, Frölich JC. Endogenous nitric oxide synthase inhibitors are responsible for the L-arginine paradox. *FEBS Lett*. 2000;478:1-3.
5. Teerlink T, Luo Z, Palm F, Wilcox CS. Cellular ADMA: Regulation and action. *Pharmacol Res*. 2009;60:448-460.
6. Siroen MP, Teerlink T, Nijveldt RJ, Prins HA, Richir MC, Van Leeuwen PA. The clinical significance of asymmetric dimethylarginine. *Annu Rev Nutr*. 2006;26:203-228.
7. Böger RH, Maas R, Schulze F, Schwedhelm E. Asymmetric dimethylarginine (ADMA) as a prospective marker of cardiovascular disease and mortality-An update on patient populations with a wide range of cardiovascular risk. *Pharmacol Res*. 2009;60:481-487.
8. Bode-Böger SM, Scalera F, Ignarro LJ. The L-arginine paradox: Importance of the L-arginine/asymmetrical dimethylarginine ratio. *Pharmacol Ther*. 2007;114:295-306.
9. Libby P, Ridker PM, Maseri A. Inflammation and atherosclerosis. *Circulation*. 2002;105:1135-1143.
10. Lind L, Siegbahn A, Hulthe J, Elmgren A. C-reactive protein and e-selectin levels are related to vasodilation in resistance, but not conductance arteries in the elderly: the prospective investigation of the Vasculature in Uppsala Seniors (PIVUS) study. *Atherosclerosis*. 2008;199:129-137.
11. Vita JA, Brennan ML, Gokce N, Mann SA, Goormastic M, Shishehbor MH, Penn MS, Keaney JF, Jr., Hazen SL. Serum myeloperoxidase levels independently predict endothelial dysfunction in humans. *Circulation*. 2004;110:1134-1139.
12. Van der Zwan LP, Teerlink T, Dekker JM, Henry RM, Stehouwer CD, Jakobs C, Heine RJ, Scheffer PG. Plasma myeloperoxidase is inversely associated with endothelium-dependent vasodilation in elderly subjects with abnormal glucose metabolism. *Metabolism*. 2010; In Press.
13. Henry RM, Kostense PJ, Spijkerman AM, Dekker JM, Nijpels G, Heine RJ, Kamp O, Westerhof N, Bouter LM, Stehouwer CD. Arterial stiffness increases with deteriorating glucose tolerance status: the Hoorn Study. *Circulation*. 2003;107:2089-2095.
14. Alberti KG, Zimmet PZ. Definition, diagnosis and classification of diabetes mellitus and its complications. Part 1: diagnosis and classification of diabetes mellitus provisional report of a WHO consultation. *Diabet Med*. 1998;15:539-553.
15. Teerlink T, Nijveldt RJ, De Jong S, Van Leeuwen PA. Determination of arginine, asymmetric dimethylarginine, and symmetric dimethylarginine in human plasma and other biological samples by high-performance liquid chromatography. *Anal Biochem*. 2002;303:131-137.

16. De Jong S, Teerlink T. Analysis of asymmetric dimethylarginine in plasma by HPLC using a monolithic column. *Anal Biochem*. 2006;353:287-289.
17. Grooteman MP, Gritters M, Wauters IM, Schalkwijk CG, Stam F, Twisk J, Ter Wee PM, Nube MJ. Patient characteristics rather than the type of dialyser predict the variability of endothelial derived surface molecules in chronic haemodialysis patients. *Nephrol Dial Transplant*. 2005;20:2751-2758.
18. Scheffer PG, Van der Zwan LP, Schindhelm RK, Vermue HP, Teerlink T. Myeloperoxidase concentrations in EDTA-plasma of healthy subjects are discordant with concentrations in heparin-plasma and serum. *Clin Biochem.* 2009;42:1490-1492.
19. Van der Zwan LP, Teerlink T, Dekker JM, Henry RM, Stehouwer CD, Jakobs C, Heine RJ, Scheffer PG. Circulating oxidized LDL: determinants and association with brachial flow-mediated dilation. *J Lipid Res.* 2009;50:342-349.
20. Levey AS, Coresh J, Greene T, Stevens LA, Zhang YL, Hendriksen S, Kusek JW, Van Lente F. Using standardized serum creatinine values in the modification of diet in renal disease study equation for estimating glomerular filtration rate. *Ann Intern Med*. 2006;145:247-254.
21. Wu G, Morris SM, Jr. Arginine metabolism: nitric oxide and beyond. *Biochem J*. 1998;336:1-17.
22. Ryoo S, Gupta G, Benjo A, Lim HK, Camara A, Sikka G, Lim HK, Sohi J, Santhanam L, Soucy K, Tuday E, Baraban E, Ilies M, Gerstenblith G, Nyhan D, Shoukas A, Christianson DW, Alp NJ, Champion HC, Huso D, Berkowitz DE. Endothelial arginase II: a novel target for the treatment of atherosclerosis. *Circ Res*. 2008;102:923-932.
23. Van Waardenburg DA, De Betue CT, Luiking YC, Engel M, Deutz NE. Plasma arginine and citrulline concentrations in critically ill children: strong relation with inflammation. *Am J Clin Nutr*. 2007;86:1438-1444.
24. Palm F, Onozato ML, Luo Z, Wilcox CS. Dimethylarginine dimethylaminohydrolase (DDAH): expression, regulation, and function in the cardiovascular and renal systems. *Am J Physiol Heart Circ Physiol*. 2007;293:H3227-H3245.
25. Kielstein JT, Salpeter SR, Bode-Böger SM, Cooke JP, Fliser D. Symmetric dimethylarginine (SDMA) as endogenous marker of renal function--a meta-analysis. *Nephrol Dial Transplant*. 2006;21:2446-2451.
26. Murray-Rust J, Leiper J, McAlister M, Phelan J, Tilley S, Santa MJ, Vallance P, McDonald N. Structural insights into the hydrolysis of cellular nitric oxide synthase inhibitors by dimethylarginine dimethylaminohydrolase. *Nat Struct Biol*. 2001;8:679-683.
27. Schindhelm RK, Van der Zwan LP, Teerlink T, Scheffer PG. Myeloperoxidase: a useful biomarker for cardiovascular disease risk stratification? *Clin Chem*. 2009;55:1462-1470.
28. Van der Zwan LP, Scheffer PG, Dekker JM, Stehouwer CD, Heine RJ, Teerlink T. Hyperglycemia and oxidative stress strengthen the association between myeloperoxidase and blood pressure. *Hypertension*. 2010;55:1366-1372.
29. Singh U, Devaraj S, Jialal I. C-reactive protein stimulates myeloperoxidase release from polymorphonuclear cells and monocytes: implications for acute coronary syndromes. *Clin Chem*. 2009;55:361-364.

30. Singh U, Devaraj S, Vasquez-Vivar J, Jialal I. C-reactive protein decreases endothelial nitric oxide synthase activity via uncoupling. *J Mol Cell Cardiol*. 2007;43:780-791.

Chapter 8

Homoarginine and arginine are antagonistically related to blood pressure

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To be submitted

Abstract

Arginine and its homologue homoarginine are substrates of nitric oxide synthase (NOS), whereas asymmetric dimethylarginine (ADMA) is a NOS inhibitor. The synthesis of nitric oxide by endothelial NOS is responsible for the vascular tone that is essential for blood pressure regulation. Blood pressure is lowered upon infusion or oral administration of arginine, but data on the relation between blood pressure and physiologic plasma levels of arginine, homoarginine, and ADMA is scarce. We investigated these relationships in a population-based cohort of men and women (n=746, aged 50 to 87).

In linear regression models adjusted for age, sex and arginine, a positive association was observed between homoarginine and systolic blood pressure (3.94 mm Hg per SD increment of homoarginine [95% CI: 2.33 to 5.55]; *P*<0.001) and diastolic blood pressure (1.82 [0.95 to 2.70]; *P*<0.001). These associations were not attenuated upon further adjustment for plasma levels of ADMA or other cardiovascular risk factors (glucose, lipids, body mass index, inflammation markers, smoking status, anti-hypertensive medication, and prior cardiovascular disease). In these models arginine was not significantly associated with systolic blood pressure (-0.92 [-2.42 to 0.58]; *P*=0.23), but a significant negative association with diastolic blood pressure was observed (-1.14 [-1.95 to -0.32]; *P*=0.006), that withstood adjustment for ADMA and other cardiovascular risk factors. ADMA was not significantly associated with either systolic or diastolic blood pressure. We conclude that the competing NOS substrates homoarginine and arginine are antagonistically associated with blood pressure.

Introduction

Production of the vasodilator nitric oxide (NO) from arginine by endothelial nitric oxide synthase (eNOS) is important in the control of blood pressure homeostasis.[1,2] As substrate of eNOS, arginine has been widely studied in the field of vascular tone maintenance.[3-5] The role of homoarginine in NO production, however, has not been widely studied.

Homoarginine differs from arginine only by an additional CH_2-group in the side chain, which makes it a homologue of arginine. Because of this structural similarity, it may act as a competing substrate or inhibitor of enzymes that use arginine as substrate. For instance, homoarginine has been identified as a substrate for eNOS.[6] On the other hand, eNOS has a significantly lower efficiency for NO production with homoarginine as substrate than with arginine,[6] potentially resulting in a reduced overall NO production.

Because eNOS is located intracellularly, transport of arginine and homoarginine across the outer cell membrane is also relevant for NOproduction. This transport is regulated by cationic amino acid transporters (CAT) of the y^+ system.[7-9] Amino acid transport by CAT strongly depends on the intracellular and local extracellular concentrations of dibasic amino acids. For example, high concentrations of homoarginine inside the cell cause a reduced uptake of arginine.[8,9] Both the reduced uptake of arginine due to high intracellular homoarginine concentrations, and the lower efficiency of eNOS with homoarginine as substrate may result in a reduced NO production, possibly leading to hypertension. To our knowledge the relation between plasma levels of homoarginine and blood pressure has never been investigated. To address this issue, we determined concentrations of arginine and homoarginine in plasma of elderly participants of a population-based cohort study, and studied biochemical and clinical correlates of these compounds and their associations with blood pressure.

Methods

Subjects

The present study was conducted in the Hoorn Study follow-up examination[10] and the Hoorn Screening Study,[11] which both are population-based studies in a white population. From the initial 822 participants, 76 subjects were excluded because of missing data on primary variables of interest, including one case with a below physiologically plausible arginine concentration (7.0 µmol/L) and another case with an excessively high ADMA concentration (over three standard deviations (SD) higher than the second highest ADMA value). In total 746 subjects (369 men and 377 women) within the age-range of 50 to 87 years remained. This population consisted of 267 subjects with a normal glucose metabolism (NGM), 190 with impaired glucose metabolism (IGM), and 289 with type 2 diabetes mellitus (T2DM), according to WHO-99 criteria.[12] The study was approved by the local ethics committee and all participants gave their written informed consent.

Blood pressure measurements

Systolic (SBP) and diastolic (DBP) blood pressure were measured using a random-zero sphygmomanometer (Hawksley-Gelman, Lansing, Sussex, United Kingdom), while subjects were in a sitting position and after they had rested for 5 minutes. Duplicate measurements were done, and mean values were used in analyses. Hypertension was defined as SBP ≥140 mm Hg or DBP ≥90 mm Hg.[13]

Biochemical analyses

Plasma concentrations of arginine, homoarginine, asymmetric dimethylarginine (ADMA), and symmetric dimethylarginine (SDMA) were determined using high-performance liquid chromatography with fluorescence detection.[14,15] The intra-assay and inter-assay coefficients of variation (CV) for all analytes were <1.5% and <4.0%, respectively. Sandwich enzyme-linked immunosorbent assays (ELISA) were used to determine myeloperoxidase (MPO) concentrations in EDTA-plasma (Mercodia, Uppsala, Sweden) with intra-assay CV of 3.9% and inter-assay CV of 5.0%,[16] and C-reactive protein (CRP) concentrations in plasma with intra-assay CV of 3.9% and inter-assay CV of 8.7%.[17] Circulating oxidized LDL (oxLDL) in plasma was determined by competitive ELISA (Mercodia, Uppsala, Sweden) and for the

determination of glycated hemoglobin (HbA1c), ion-exchange high-performance liquid chromatography (reference range 4.3-6.1%) was used on a modular monitoring system (Bio-Rad, Veenendaal, The Netherlands). Glucose, HDL-cholesterol (HDL-c) and triglycerides were measured by standard enzymatic methods (Roche, Mannheim, Germany), while LDL-cholesterol (LDL-c) concentration was determined with a direct method by the “N-geneous” assay (GenZyme, Cambridge, MA, USA). With this method, triglyceride concentrations up to 13.5 mmol/L do not interfere with measurement of LDL-c.

Renal function and cardiovascular disease

Estimated glomerular filtration rate (eGFR) was determined by the 4-variable MDRD formula as described by Levey et al.,[18] and microalbuminuria was defined as urinary albumin/creatinine ratio≥2.0 mg/mmol. Prior cardiovascular diseas e (CVD) was defined as either Minnesota Code 1.1 to 1.3, 4.1 to 4.3, 5.1 to 5.3, or 7.1 on the electrocardiogram; coronary bypass operation or angioplasty; an ankle-brachial blood pressure index <0.9 in either leg; peripheral arterial bypass; or amputation for atherosclerotic disease.

Statistics

Arginine, homoarginine, and ADMA concentrations were normally distributed. Data is presented as mean with standard deviation (SD) or, for skewed variables, median and interquartile range. The skewed variables were log-transformed prior to linear regression analyses. Student’s t-test was applied for comparison of variables between two groups.

Both linear trend analyses, and analysis of arginine and homoarginine correlates with linear regression, were adjusted for age and sex. In multivariable regression models with arginine or homoarginine as dependent variables, age and sex were added (enter modus) to all models, whereas other variables were entered stepwise (stepwise modus), and only if a *P*-value <0.1 for age- and sex-adjusted association was found. To avoid co-linearity in multivariable analyses, fasting glucose concentration, instead of the closely related HbA1c, was chosen as a measure of glucose metabolism.

Associations of homoarginine and arginine with blood pressure were studied by multivariable linear regression analysis. Regression coefficients were expressed as change in blood pressure (mm Hg) per SD increase of homoarginine or arginine. While examining for possible effect modification, the models were adjusted for age, sex, and homoarginine or arginine. Data was analyzed using SPSS software, version 17 (SPSS Inc., Chicago, IL). A two-tailed *P*-value <0.05 was considered to indicate statistical significance.

Results

Subject characteristics

Subject characteristics are presented in Table 1. Arginine concentrations did not significantly differ (*P*=0.13) between men (95.0±15.6 µmol/L) and women (93.3±15.4 µmol/L), whereas homoarginine concentrations were higher in men (1.67±0.53 µmol/L) than in women (1.33±0.46 µmol/L; *P*<0.001). Homoarginine (*P*=0.27) and arginine concentrations (*P*=0.65) did not differ significantly between subjects with NGM and IGM, but compared to subjects with NGM subjects with T2DM had higher levels of homoarginine (1.60±0.57 versus 1.42±0.50 µmol/L; *P*<0.001) and lower levels of arginine (91.7±15.5 versus 95.3±14.4 µmol/L; *P*=0.006).

Table 1. Subject characteristics

Variable	Units	
N		746
Male sex	%	49
Age	years	68.8 (7.2)
Arginine	µmol/L	94.1 (15.6)
Homoarginine	µmol/L	1.50 (0.52)
ADMA	µmol/L	0.449 (0.058)
SDMA	µmol/L	0.498 (0.110)
Systolic blood pressure	mm Hg	142 (20)
Diastolic blood pressure	mm Hg	83 (11)
Hypertension	%	58
Glucose*	mmol/L	6.1 (5.5-7.0)
HbA1c	%	6.1 (0.8)
Glomerular filtration rate	$ml \cdot min^{-1}/1.73\ m^2$	60.5 (10.6)
Microalbuminuria	%	15
LDL-cholesterol	mmol/L	3.6 (0.9)
HDL-cholesterol	mmol/L	1.38 (0.40)
Triglycerides*	mmol/L	1.4 (1.0-1.9)
Oxidized LDL	U/L	64.8 (15.2)
Myeloperoxidase*	µg/L	56.1 (47.4-67.0)
C-reactive protein*	mg/L	2.20 (1.06-4.68)
Current smoking	%	15
Antihypertensive medication use	%	39
Lipid lowering medication use	%	18
Body mass index	kg/m^2	27.7 (4.2)
Prior cardiovascular disease	%	49

Data is presented as means (SD). *Variables with skewed distributions are depicted as medians (interquartile range). ADMA, asymmetric dimethylarginine; SDMA, symmetric dimethylarginine.

Correlates of homoarginine and arginine

In the age and sex-adjusted analyses a significant positive association (P<0.001) between arginine and homoarginine was found (Table 2). Furthermore, homoarginine was positively associated with SBP, DBP, fasting glucose, HbA1c, and BMI, while it was negatively associated with age, microalbuminuria, and current smoking status. In contrast, arginine was negatively associated with fasting glucose and BMI, but positively associated with current smoking status. Variables that were significantly associated only with arginine, were ADMA and HDL-c, which were both positive correlates, and triglycerides, MPO, and CRP that were negative correlates.

To determine the independent correlates of homoarginine and arginine multivariable linear regression analyses were performed. A model containing age, sex, BMI, glucose, current smoking status, and microalbuminuria as independent variables explained about eighteen percent of the variation in homoarginine concentrations. Eight percent of the variation in arginine concentrations was explained by a regression model containing age, sex, smoking status, BMI, and CRP as independent variables.

Table 2. Biochemical and clinical correlates of homoarginine and arginine

		Homoarginine		Arginine	
Variables	Units	Standardized β†	*P*	Standardized β†	*P*
Age	years	-0.169	<0.001	0.010	0.79
Sex	male	0.312	<0.001	0.057	0.12
Homoarginine	µmol/L	-	-	0.347	<0.001
Arginine	µmol/L	0.304	<0.001	-	-
ADMA	µmol/L	0.014	0.69	0.276	<0.001
SDMA	µmol/L	-0.050	0.18	0.043	0.28
Systolic blood pressure	mm Hg	0.161	<0.001	0.014	0.71
Diastolic blood pressure	mm Hg	0.115	0.001	-0.054	0.14
Hypertension	y/n	0.135	<0.001	0.020	0.58
Glucose*	mmol/L	0.121	<0.001	-0.116	0.002
HbA1c	%	0.081	0.018	-0.086	0.019
Glomerular filtration rate	$ml \cdot min^{-1}/1.73\ m^2$	-0.020	0.60	-0.049	0.23
Microalbuminuria	y/n	-0.071	0.041	-0.057	0.12
LDL-cholesterol	mmol/L	-0.058	0.09	0.049	0.19
HDL-cholesterol	mmol/L	-0.046	0.22	0.098	0.014
Triglycerides*	mmol/L	0.050	0.15	-0.099	0.007
Oxidized LDL	U/L	0.014	0.68	-0.010	0.79
Myeloperoxidase*	µg/L	0.001	0.97	-0.087	0.018
C-reactive protein*	mg/L	-0.025	0.46	-0.213	<0.001
Current smoker	y/n	-0.102	0.003	0.143	<0.001
Antihypertensive medication	y/n	0.027	0.44	-0.050	0.18
Lipid lowering medication	y/n	0.066	0.06	0.003	0.94
Body mass index	kg/m^2	0.145	<0.001	-0.166	<0.001
Prior cardiovascular disease	y/n	0.009	0.81	0.003	0.94

*Skewed variables were log-transformed prior to analysis. †Standardized regression coefficients derived from age- and sex-adjusted linear regression analysis. Abbreviations: ADMA, asymmetric dimethylarginine; SDMA, symmetric dimethylarginine.

Associations of homoarginine and arginine with blood pressure

Sex was neither an effect modifying factor on the association of homoarginine with blood pressure (SBP: *P*=0.2; DBP: *P*=0.4) nor on the association between arginine and blood pressure (SBP: *P*=0.2; DBP: *P*=0.1). Analyses were, therefore, combined for both sexes. Since age and sex are well-known risk factors for CVD, and many other tested variables may depend on them, all linear regression analyses were adjusted for age and sex. As illustrated in Figure 1, an increasing linear trend

was observed for both SBP and DBP across increasing sex-specific tertiles of homoarginine.

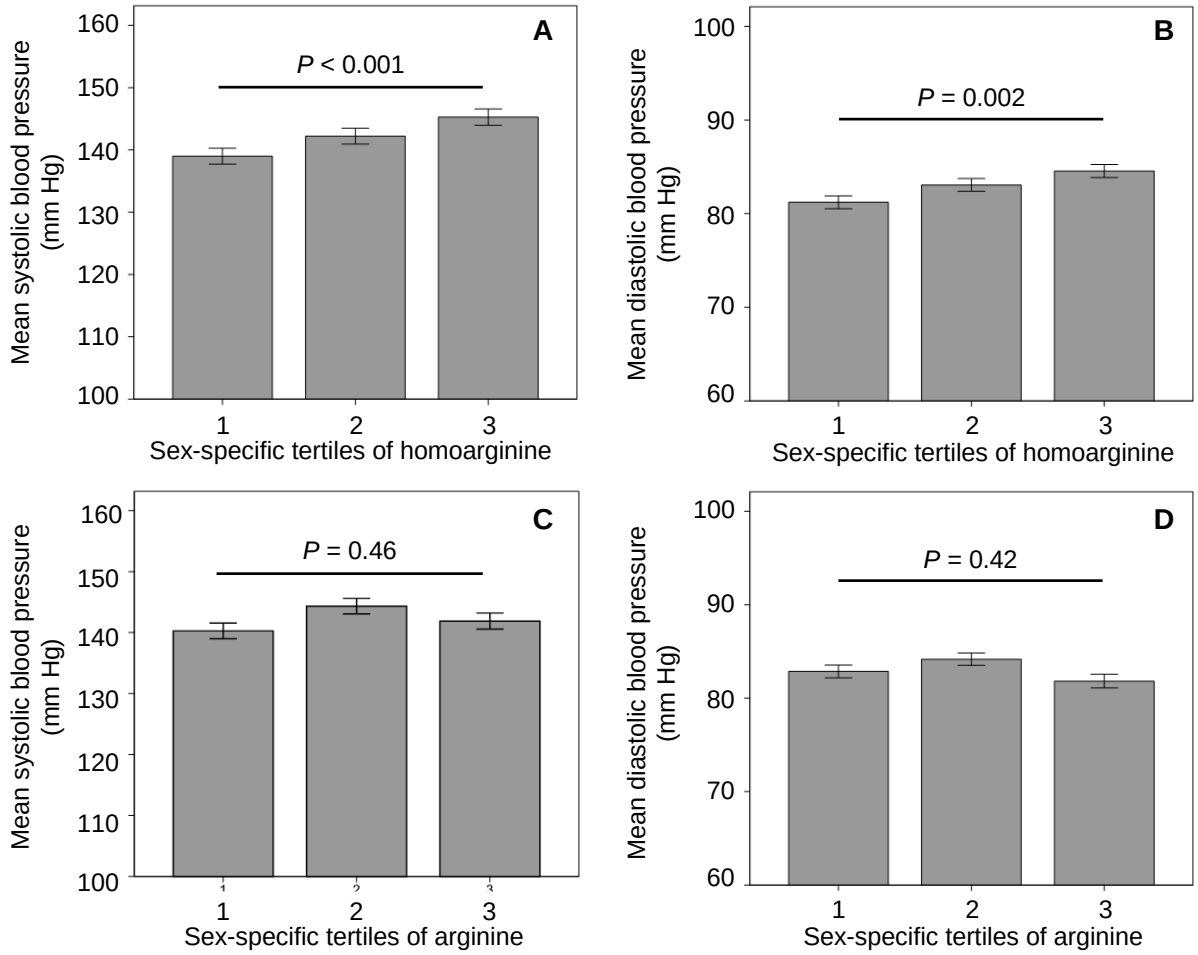

Figure 1. Mean blood pressure across sex-specific tertiles of homoarginine and arginine
Significant linear trends in SBP (panel A) and DBP (panel B) across sex-specific tertiles of homoarginine were observed, whereas there was no significant trends in SBP (panel C) and DBP (panel D) across sex-specific tertiles of arginine. Data is presented as mean pressure (mm Hg) and error bars represent SEM.

In contrast, no significant trends were found for either SBP or DBP across increasing sex-specific tertiles of arginine, confirming the lack of significant associations between blood pressure and arginine expressed as continuous variable (Table 2). After adjustment for homoarginine concentrations, however, a significant and inverse association between arginine and DBP was found. Therefore, the age and sex-adjusted associations of the arginine/homoarginine ratio with SBP and DBP were tested. The arginine/homoarginine ratio was significantly associated (P<0.001) with both SBP (-3.87 mm Hg per SD increment of arginine/homoarginine [95%CI: -5.39 to

-2.35]) and DBP (-1.69 [-2.52 to -0.86]). In multivariable analyses positive associations between homoarginine and SBP (Table 3), and between homoarginine and DBP (Table 4) were observed. The associations for homoarginine strengthened considerably after adjustment for age and sex (model 1), and were slightly further strengthened after additional adjustment for arginine (model 2). Adjustment for other correlates did not alter the strength of the associations, except for the additional adjustment for glucose or BMI, which led to a slight decrease in the strength of the associations of homoarginine with either SBP or DBP.

Table 3. Linear regression models for the relations between homoarginine and arginine with systolic blood pressure

	Homoarginine		Arginine	
Model	**Beta**[‡]	***P***	**Beta**[‡]	***P***
Crude	2.25 (0.79 to 3.71)	0.003	0.28 (-1.19 to 1.74)	0.71
Model 1 = age and sex-adjusted	3.62 (2.10 to 5.14)	<0.001	0.27 (-1.17 to 1.71)	0.71
Model 2 = model 1 + arginine or homoarginine[†]	3.94 (2.33 to 5.55)	<0.001	-0.92 (-2.42 to 0.58)	0.23
Model 2 + ADMA	3.87 (2.26 to 5.48)	<0.001	-0.68 (-2.24 to 0.88)	0.39
Model 2 + glucose[*]	3.30 (1.69 to 4.92)	<0.001	-0.36 (-1.86 to 1.15)	0.64
Model 2 + microalbuminuria	4.06 (2.45 to 5.67)	<0.001	-0.85 (-2.35 to 0.64)	0.26
Model 2 + LDL-cholesterol	3.94 (2.32 to 5.56)	<0.001	-0.91 (-2.42 to 0.59)	0.23
Model 2 + HDL-cholesterol	3.81 (2.20 to 5.42)	<0.001	-0.74 (-2.25 to 0.76)	0.33
Model 2 + triglycerides[*]	3.78 (2.17 to 5.38)	<0.001	-0.65 (-2.16 to 0.86)	0.40
Model 2 + myeloperoxidase[*]	3.87 (2.27 to 5.47)	<0.001	-0.72 (-2.21 to 0.78)	0.35
Model 2 + C-reactive protein[*]	3.83 (2.23 to 5.43)	<0.001	-0.42 (-1.95 to 1.11)	0.59
Model 2 + current smoker	3.69 (2.06 to 5.33)	<0.001	-0.65 (-2.19 to 0.88)	0.40
Model 2 + antihyp. med.	3.78 (2.18 to 5.37)	<0.001	-0.72 (-2.21 to 0.77)	0.34
Model 2 + lip. low. med.	3.96 (2.34 to 5.58)	<0.001	-0.92 (-2.43 to 0.58)	0.23
Model 2 + body mass index	3.06 (1.43 to 4.69)	<0.001	-0.07 (-1.59 to 1.45)	0.93
Model 2 + prior CVD	3.93 (2.29 to 5.57)	<0.001	-0.92 (-2.45 to 0.61)	0.24

[*] Skewed variables were log-transformed prior to analyses. [†] To obtain model 2, model 1 was additionally adjusted for arginine in case of the association between homoarginine and SBP, and for homoarginine in case of the association between arginine and SBP. [‡] Beta is the change of SBP in mm Hg per SD increment of homoarginine or arginine. Abbreviations: ADMA, asymmetric dimethylarginine; Antihyp. med, anti hypertensive medication; lip. low. med, lipid lowering medication; prior CVD, prior cardiovascular disease.

Table 4. Linear regression models for the relations between homoarginine and arginine with diastolic blood pressure

	Homoarginine		Arginine	
Model	**Beta**[‡]	***P***	**Beta**[‡]	***P***
Crude	1.57 (0.79 to 2.34)	<0.001	-0.57 (-1.35 to 0.22)	0.16
Model 1 = age and sex-adjusted	1.43 (0.59 to 2.26)	<0.001	-0.59 (-1.37 to 0.19)	0.14
Model 2 = model 1 + arginine or homoarginine[†]	1.82 (0.95 to 2.70)	<0.001	-1.14 (-1.95 to -0.32)	0.006
Model 2 + ADMA	1.83 (0.95 to 2.71)	<0.001	-1.17 (-2.02 to -0.32)	0.007
Model 2 + glucose[*]	1.49 (0.61 to 2.38)	0.001	-0.85 (-1.67 to -0.03)	0.043
Model 2 + microalbuminuria	1.91 (1.04 to 2.78)	<0.001	-1.09 (-1.90 to -0.28)	0.009
Model 2 + LDL-cholesterol	1.82 (0.94 to 2.70)	<0.001	-1.13 (-1.96 to -0.31)	0.007
Model 2 + HDL-cholesterol	1.75 (0.87 to 2.63)	<0.001	-1.04 (-1.86 to -0.22)	0.013
Model 2 + triglycerides[*]	1.69 (0.81 to 2.56)	<0.001	-0.97 (-1.78 to -1.48)	0.021
Model 2 + myeloperoxidase[*]	1.79 (0.92 to 2.66)	<0.001	-1.05 (-1.86 to -0.23)	0.012
Model 2 + C-reactive protein[*]	1.79 (0.91 to 2.66)	<0.001	-0.98 (-1.82 to -0.15)	0.021
Model 2 + current smoker	1.68 (0.79 to 2.57)	<0.001	-0.98 (-1.82 to -0.15)	0.021
Model 2 + antihyp. med.	1.74 (0.87 to 2.61)	<0.001	-1.04 (-1.85 to -0.22)	0.012
Model 2 + lip. low. med.	1.83 (0.95 to 2.71)	<0.001	-1.14 (-1.96 to -0.32)	0.007
Model 2 + body mass index	1.19 (0.31 to 2.07)	0.008	-0.53 (-1.35 to 0.29)	0.21
Model 2 + prior CVD	1.82 (0.92 to 2.72)	<0.001	-1.14 (-1.97 to -0.30)	0.008

[*] Skewed variables were log-transformed prior to analyses. [†] To obtain model 2, model 1 was additionally adjusted for arginine in case of the association between homoarginine and DBP, and for homoarginine in case of the association between arginine and DBP. [‡] Beta is the change of DBP in mm Hg per SD increment of homoarginine or arginine. Abbreviations: ADMA, asymmetric dimethylarginine; Antihyp. med, anti hypertensive medication; lip. low. med, lipid lowering medication; prior CVD, prior cardiovascular disease.

No effect modification was observed on the association of homoarginine with blood pressure, when factors like hypertensive medication use, microalbuminuria, IGM, and T2DM were taken into account.

Finally, ADMA, the endogenous inhibitor of NOS, was tested in relation to blood pressure, but neither in the total population, nor when glucose metabolism or hypertensive medicine use was taken into account, an association between ADMA and blood pressure was found.

Discussion

In the present study a robust positive association between plasma homoarginine concentrations and both SBP and DBP was observed, which was independent of traditional cardiovascular risk factors, like smoking, use of antihypertensive medication, prior CVD, and cholesterol levels. Homoarginine may, therefore, be important in blood pressure control.

Potential mechanisms for homoarginine synthesis and degradation

In Figure 2 an overview is given of the potential mechanisms for homoarginine synthesis and degradation. Homoarginine may be synthesized by two enzymes, L-arginine:glycine amidinotransferase (AGAT) or argininosuccinate lyase (ASL). AGAT is a key enzyme in the synthesis of creatine. It transfers an amidino-group from arginine to glycine resulting in the formation of ornithine and guanidinoacetate (GAA), which is subsequently converted into creatine by guanidinoacetate methyl transferase (GAMT).[19] It has been suggested that AGAT may be used alternatively in homoarginine synthesis, using lysine as acceptor of the amidino-group instead of glycine, which results in the formation of ornithine and homoarginine.[20,21] In creatine synthesis GAA is directly converted into creatine by GAMT, whereas homoarginine remains available for the re-formation of lysine using AGAT. The other possible route for homoarginine synthesis is through an alternative urea cycle.[22-26] In this cycle arginine, ornithine, citrulline, and argininosuccinate are replaced by homoarginine, lysine, homocitrulline, and homoargininosuccinate, respectively. Homocitrulline is converted into homoargininosuccinate by argininosuccinate synthase (ASS), which is then converted by ASL into homoarginine. Aside from the formation of homoarginine, this alternative urea cycle also presents a route for the degradation of homoarginine through arginase, resulting in lysine and urea. Additional support for this potential cycle is provided by the increased homoarginine concentrations in plasma and urine observed in hyperlysinemea.[25]

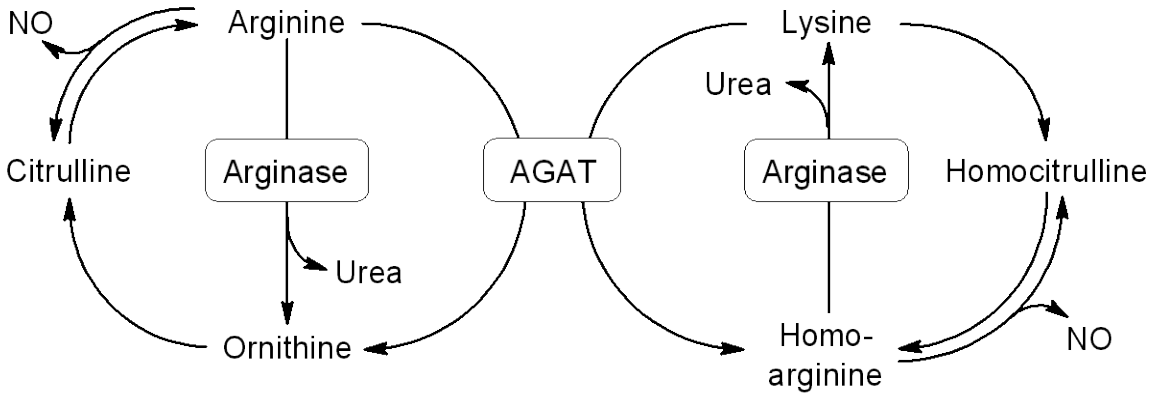

Figure 2. Mechanisms of homoarginine synthesis and degradation
Arginine is synthesized in the urea cycle (left). Homoarginine can be synthesized by the same set of enzymes, but with replacement of arginine, ornithine, and citrulline by homoarginine, lysine, and homocitrulline, respectively (right). A second pathway by which homoarginine can be synthesized, involves a transamidination reaction, with arginine as donor and lysine as acceptor of an amidinogroup (center). This reaction is catalyzed by L-arginine:glycine amidinotransferase (AGAT), the rate-limiting enzyme in the synthesis of creatine. Pathways for homoarginine degradation include hydrolysis by arginase into lysine and urea, and conversion by nitric oxide synthase into homocitrulline and nitric oxide.

Arginine and homoarginine are antagonistically associated with blood pressure

eNOS is able to use homoarginine as substrate, leading to NO formation and a subsequent reduction in blood pressure. However, homoarginine is a less efficient substrate for eNOS than arginine, which may lead to a decreased overall NO availability and a subsequent increased blood pressure.

In the present study we found that homoarginine was positively associated with arginine. Whereas homoarginine was independently and positively associated with both DBP and SBP, arginine was only negatively associated with DBP when accounting for homoarginine concentrations. In addition, significant correlates of homoarginine were either different from those of arginine or, when correlates were shared by arginine and homoarginine, were of opposite sign. Homoarginine may, therefore, be an antagonist of arginine in blood pressure control.

The positive association between homoarginine and blood pressure may be explained by several factors that in conjunction lead to the diminished availability of NO. Firstly, arginine and homoarginine compete for cell entry by CAT. High homoarginine concentrations outside the cell will lead to reduced arginine uptake, which is necessary for sufficient NO production. Secondly, homoarginine is an alternative substrate for NOS. Although NO is produced by NOS with homoarginine

as substrate, the efficiency is considerably lower, which will also lead to reduced NO synthesis. Together, the diminished arginine uptake and the low efficiency of NOS with homoarginine may cause altered blood pressure homeostasis through reduction in NO.

In support of our observations Kakoki et al.[27] observed an increased NO production in kidney cells after arginine supplementation and a decreased NO production after supplementation of homoarginine. However, a study in salt-sensitive hypertensive mice by Chen et al.[28] showed a blood pressure reduction after administration of either arginine or homoarginine. The observations of Chen may be specific for salt-sensitive hypertensive mice, because in their study salt-sensitive mice showed increased arginine concentrations when fed a high salt diet, while salt-resistant hypertensive mice did not, indicating distinct differences. Furthermore, the high dose of homoarginine used in the experiments by Chen may also have resulted in a net different effect on blood pressure.

Arginine and ADMA in relation to blood pressure

Some studies showed a protective effect of arginine against CVD, and others showed a positive association between ADMA and CVD.[3,5,29-31] In contrast, there are also studies in which no association between arginine and vascular function was found.[32] In the present study no association was found between ADMA and blood pressure. In literature several explanations are suggested for the lack of association of ADMA and arginine with vascular function. These include the use of antihypertensive medicine and impaired kidney function.[32] However, adjustment for these factors did not result in significant associations of ADMA or arginine with blood pressure. Only after adjustment for homoarginine, a significant association between arginine and DBP was observed, which may be explained by the antagonistic association between homoarginine and arginine. Significant associations between arginine and SBP were not observed. The limited association between arginine and blood pressure is probably the result of other arginine-consuming enzymes that are not involved in blood pressure control, like in the formation of creatine. This, combined with processes that influence ADMA concentrations (methylation, proteolysis, renal clearance, and degradation by dimethylarginine dimethylaminohydrolase)[7] may also explain the lack of association between ADMA and

blood pressure, since the arginine/ADMA ratio is a more direct measure of NO production by eNOS.

Role of traditional risk factors in the association of homoarginine with blood pressure

In the assessment of the relation between homoarginine and blood pressure, known blood pressure raising variables were taken into account. BMI and fasting glucose concentrations were positive correlates of homoarginine. However, age and smoking were negative correlates of homoarginine. Possibly this is caused by the high age range of our study-population. Both aging and smoking lessen the elasticity of the arteries, which also causes an increased blood pressure. Previously, we studied the effect of oxidative stress on blood pressure, and reported the positive association of MPO with blood pressure.[33] In the present study homoarginine was not associated with markers of inflammation or oxidative stress, i.e. oxLDL, MPO, or CRP. The association of homoarginine with blood pressure is, therefore, likely to reflect processes in blood pressure control, other than inflammation, which is reflected by the association between MPO and blood pressure. Finally, the association of homoarginine with kidney function, assessed by eGFR and prevalence of microalbuminuria, was studied. A significant association with eGFR was not found. However, microalbuminuria was negatively associated with homoarginine, which might be explained by the fact that synthesis of homoarginine takes place in the kidneys, and is reduced in kidney disease.

Study limitations

First, the study cohort was designed to investigate glucose metabolism in elderly subjects. In general and in this study a high age and poor glycemic control coincide with an increased prevalence of hypertension. Since subjects were elderly and subjects with IGM and T2DM were over represented, associations may be different in the general population. Nonetheless, we did not observe effect modification on the association of homoarginine with blood pressure by states of glucose metabolism. Second, little is known about the role of homoarginine in humans. Although a large number of variables were used for the adjustment of the association between homoarginine and blood pressure, residual confounding cannot be excluded.

Perspectives

Using current antihypertensive medicine, a large group of hypertensive patients is still unable to lower their blood pressure to set targets.[34] Hence, a clear need for novel therapies and drugs exists. The design of more effective drugs, may require better understanding of the molecular mechanisms involved in hypertension. The observation that homoarginine plays a role in human blood pressure homeostasis is new. This implies that measurement of homoarginine in future research may improve insight into underlying causes of hypertension, which may be useful in developing new antihypertensive drugs.

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Conflicts of interests and disclosures

None.

References

1. Török J. Participation of nitric oxide in different models of experimental hypertension. *Physiol Res*. 2008;57:813-825.
2. Maxwell AJ. Mechanisms of dysfunction of the nitric oxide pathway in vascular diseases. *Nitric Oxide*. 2002;6:101-124.
3. Sato H, Zhao ZQ, McGee DS, Williams MW, Hammon JW, Jr., Vinten-Johansen J. Supplemental L-arginine during cardioplegic arrest and reperfusion avoids regional postischemic injury. *J Thorac Cardiovasc Surg*. 1995;110:302-314.
4. Lin CC, Tsai WC, Chen JY, Li YH, Lin LJ, Chen JH. Supplements of l-arginine attenuate the effects of high-fat meal on endothelial function and oxidative stress. *Int J Cardiol*. 2008;127:337-341.
5. Ast J, Jablecka A, Bogdanski P, Smolarek I, Krauss H, Chmara E. Evaluation of the antihypertensive effect of L-arginine supplementation in patients with mild hypertension assessed with ambulatory blood pressure monitoring. *Med Sci Monit*. 2010;16:CR266-CR271.
6. Moali C, Boucher JL, Sari MA, Stuehr DJ, Mansuy D. Substrate specificity of NO synthases: detailed comparison of L-arginine, homo-L-arginine, their N omega-hydroxy derivatives, and N omega-hydroxynor-L-arginine. *Biochemistry*. 1998;37:10453-10460.
7. Teerlink T, Luo Z, Palm F, Wilcox CS. Cellular ADMA: Regulation and action. *Pharmacol Res*. 2009;60:448-460.
8. Inoue Y, Bode BP, Beck DJ, Li AP, Bland KI, Souba WW. Arginine transport in human liver. Characterization and effects of nitric oxide synthase inhibitors. *Ann Surg*. 1993;218:350-362.
9. White MF, Gazzola GC, Christensen HN. Cationic amino acid transport into cultured animal cells. I. Influx into cultured human fibroblasts. *J Biol Chem*. 1982;257:4443-4449.
10. Mooy JM, Grootenhuis PA, De Vries H, Valkenburg HA, Bouter LM, Kostense PJ, Heine RJ. Prevalence and determinants of glucose intolerance in a Dutch caucasian population. The Hoorn Study. *Diabetes Care*. 1995;18:1270-1273.
11. Spijkerman AM, Adriaanse MC, Dekker JM, Nijpels G, Stehouwer CD, Bouter LM, Heine RJ. Diabetic patients detected by population-based stepwise screening already have a diabetic cardiovascular risk profile. *Diabetes Care*. 2002;25:1784-1789.
12. Alberti KG, Zimmet PZ. Definition, diagnosis and classification of diabetes mellitus and its complications. Part 1: diagnosis and classification of diabetes mellitus provisional report of a WHO consultation. *Diabet Med*. 1998;15:539-553.
13. Chobanian AV, Bakris GL, Black HR, Cushman WC, Green LA, Izzo JL, Jr., Jones DW, Materson BJ, Oparil S, Wright JT, Jr., Roccella EJ. Seventh report of the Joint National Committee on Prevention, Detection, Evaluation, and Treatment of High Blood Pressure. *Hypertension*. 2003;42:1206-1252.
14. Teerlink T, Nijveldt RJ, De Jong S, Van Leeuwen PA. Determination of arginine, asymmetric dimethylarginine, and symmetric dimethylarginine in human plasma and other biological samples by high-performance liquid chromatography. *Anal Biochem*. 2002;303:131-137.

15. De Jong S, Teerlink T. Analysis of asymmetric dimethylarginine in plasma by HPLC using a monolithic column. *Anal Biochem*. 2006;353:287-289.
16. Scheffer PG, Van der Zwan LP, Schindhelm RK, Vermue HP, Teerlink T. Myeloperoxidase concentrations in EDTA-plasma of healthy subjects are discordant with concentrations in heparin-plasma and serum. *Clin Biochem*. 2009;42:1490-1492.
17. Grooteman MP, Gritters M, Wauters IM, Schalkwijk CG, Stam F, Twisk J, Ter Wee PM, Nube MJ. Patient characteristics rather than the type of dialyser predict the variability of endothelial derived surface molecules in chronic haemodialysis patients. *Nephrol Dial Transplant*. 2005;20:2751-2758.
18. Levey AS, Coresh J, Greene T, Stevens LA, Zhang YL, Hendriksen S, Kusek JW, Van Lente F. Using standardized serum creatinine values in the modification of diet in renal disease study equation for estimating glomerular filtration rate. *Ann Intern Med*. 2006;145:247-254.
19. Wyss M, Kaddurah-Daouk R. Creatine and creatinine metabolism. *Physiol Rev*. 2000;80:1107-1213.
20. Srivenugopal KS, Adiga PR. Partial purification and properties of a transamidinase from Lathyrus sativus seedlings. Involvement in homoarginine metabolism and amine interconversions. *Biochem J*. 1980;189:553-560.
21. Ryan WL, Johnson RJ, Dimari S. Homoarginine synthesis by rat kidney. *Arch Biochem Biophys*. 1969;131:521-526.
22. Kato T, Sano M, Mizutani N, Hayakawa C. Homocitrullinuria and homoargininuria in hyperargininaemia. *J Inherit Metab Dis*. 1988;11:261-265.
23. Derave W, Marescau B, Van den Eede, Eijnde BO, De Deyn PP, Hespel P. Plasma guanidino compounds are altered by oral creatine supplementation in healthy humans. *J Appl Physiol*. 2004;97:852-857.
24. Ryan WL, Barak AJ, Johnson RJ. Lysine, homocitrulline, and homoarginine metabolism by the isolated perfused rat liver. *Arch Biochem Biophys*. 1968;123:294-297.
25. Woody NC, Ong EB. Paths of lysine degradation in patients with hyperlysinemia. *Pediatrics*. 1967;40:986-992.
26. Ryan WL, Wells IC. Homocitrulline and homoarginine synthesis fron lysine. *Science*. 1964;144:1122-1127.
27. Kakoki M, Kim HS, Arendshorst WJ, Mattson DL. L-Arginine uptake affects nitric oxide production and blood flow in the renal medulla. *Am J Physiol Regul Integr Comp Physiol*. 2004;287:R1478-R1485.
28. Chen PY, Sanders PW. Role of nitric oxide synthesis in salt-sensitive hypertension in Dahl/Rapp rats. *Hypertension*. 1993;22:812-818.
29. Böger RH, Sullivan LM, Schwedhelm E, Wang TJ, Maas R, Benjamin EJ, Schulze F, Xanthakis V, Benndorf RA, Vasan RS. Plasma Asymmetric Dimethylarginine and Incidence of Cardiovascular Disease and Death in the Community. *Circulation*. 2009;119:1592-1600.

30. Richir MC, van Lambalgen AA, Teerlink T, Wisselink W, Bloemena E, Prins HA, de Vries TP, van Leeuwen PA. Low arginine/asymmetric dimethylarginine ratio deteriorates systemic hemodynamics and organ blood flow in a rat model. *Crit Care Med*. 2009;37:2010-2017.
31. Wanby P, Teerlink T, Brudin L, Brattstrom L, Nilsson I, Palmqvist P, Carlsson M. Asymmetric dimethylarginine (ADMA) as a risk marker for stroke and TIA in a Swedish population. *Atherosclerosis*. 2006;185:271-277.
32. Bode-Böger SM, Scalera F, Ignarro LJ. The L-arginine paradox: Importance of the L-arginine/asymmetrical dimethylarginine ratio. *Pharmacol Ther*. 2007;114:295-306.
33. Van der Zwan LP, Scheffer PG, Dekker JM, Stehouwer CD, Heine RJ, Teerlink T. Hyperglycemia and oxidative stress strengthen the association between myeloperoxidase and blood pressure. *Hypertension*. 2010;55:1366-1372.
34. Singer GM, Izhar M, Black HR. Goal-oriented hypertension management: translating clinical trials to practice. *Hypertension*. 2002;40:464-469.

Chapter 9

Summary and concluding remarks

Summary

Although major progress has been made in the treatment and prevention of cardiovascular disease (CVD), CVD-related mortality is still a major cause of death in Western societies. Retention of LDL-cholesterol in the vessel wall is an early step in atherosclerosis development. In contrast to native LDL particles, which are not taken up by macrophages, oxidatively-modified LDL particles can be taken up in excess by macrophages, resulting in their transformation into foam cells. Accumulation of foam cells in the vessel wall leads to formation of fatty streaks, which ultimately may evolve to atherosclerotic plaques. Acute cardiovascular events are initiated by rupture of unstable atherosclerotic plaques. Over the past decades evidence has accumulated that atherosclerosis is precipitated by dyslipidemia and hyperglycemia. In addition, inflammation, oxidative stress, and impaired nitric oxide production are also involved in all stages of atherosclerosis, from early endothelial dysfunction to late stage plaque rupture. To gain more insight in how oxidative stress, inflammation and nitric oxide formation relate to atherosclerosis, we investigated a selection of biomarkers reflecting these processes. All studies were performed in a population-based cohort of elderly subjects stratified for glucose tolerance (The Hoorn Study).

In **chapter 2** we investigated the determinants of plasma oxidized low-density lipoprotein (oxLDL) and the association of this oxidative stress marker with flow-mediated dilation (FMD) of the brachial artery, which reflects endothelial function and is an early marker of atherosclerosis. It is likely that the concentration of oxLDL depends not only on the degree of oxidative stress, but also on the amount of substrate available for oxidation, i.e. the number of LDL particles. In line with this notion, we observed that oxLDL was strongly correlated with the plasma concentration of LDL-cholesterol and apolipoprotein B-100 (apoB100). The strong correlation between these variables makes it difficult to disentangle their independent contributions to CVD risk and prompted us to test the hypothesis that the oxLDL / LDL-cholesterol ratio and the oxLDL / apoB100 ratio are more informative than the separate variables. In support of this hypothesis, brachial FMD was not significantly related to plasma levels of oxLDL, LDL-cholesterol, or apoB100; whereas a negative association between FMD and the oxLDL / LDL-cholesterol and LDL / apoB100 ratios was observed. The latter association withstood adjustment for age, sex, Framingham risk score, renal function and obesity. We concluded from this study that correction of

oxLDL for LDL particle number may improve the clinical usefulness of oxLDL measurement.

In **chapter 3** the use of myeloperoxidase (MPO) as biomarker for cardiovascular risk stratification is reviewed. To this end, results of published cross-sectional studies, case-control studies, and prospective cohort studies investigating the relation between MPO and CVD were discussed. Most of the studies showed a significant positive association between high plasma concentrations of MPO and CVD-related morbidity and/or mortality. Of note, the strongest associations were observed in populations with acute cardiovascular symptoms. In several studies measurement of MPO was associated with improved risk stratification above and beyond risk stratification obtained with markers used in routine clinical practice, such as troponin-T and CK-MB. This chapter also summarizes the mechanisms by which MPO may contribute to CVD. MPO plays a beneficial role in host defense by producing hypochlorous acid and other highly reactive compounds that kill pathogenic microorganisms. In the vascular system, however, these MPO-derived reactive substances may interfere with endothelial function and lead to structural damage. In addition, MPO reduces the bioavailability of nitric oxide, is involved in oxidation of LDL and impairment of HDL function, and contributes to plaque thinning and rupture by activating metalloproteinases. Overall, both in vitro experiments and pathophysiological observations support a causal role of MPO in initiating CVD and precipitating acute cardiovascular events. Additionally, attention is paid to pitfalls in the laboratory analysis of MPO. This issue is further expanded on in **chapter 4,** where matched serum, heparin-plasma, and EDTA-plasma of healthy volunteers were compared for MPO concentrations. In heparin-plasma MPO concentrations were twice as high as in EDTA-plasma and in serum even four times higher MPO concentrations were found. More importantly, MPO concentrations in EDTA-plasma were not significantly correlated with MPO concentrations in heparin-plasma or serum. We argued that ex vivo release of MPO from leukocytes induced by heparin or due to coagulation is most likely responsible for these differences in MPO concentrations. Accordingly, we recommended to use EDTA-plasma for the measurement of MPO, because these values may most accurately reflect the concentration of MPO in the circulation.

In **chapter 5** the correlates of the EDTA-plasma MPO concentration and the association of MPO concentration with FMD of the brachial artery were investigated.

In a multivariable linear regression model, daily vitamin C intake and plasma levels of C-reactive protein (CRP) were significant independent determinants of plasma MPO concentration, together explaining approximately 9% of its variation. We hypothesized that the association between MPO and FMD would be stronger in subjects with elevated glucose concentrations, because hyperglycemia is associated with increased levels of hydrogen peroxide, an essential cosubstrate of MPO. Indeed, a significant inverse association between MPO and FMD was found in subjects with abnormal glucose metabolism (impaired glucose metabolism and type 2 diabetes mellitus combined), but not in subjects with normoglycemia. This negative association was not attenuated after adjustment for established CVD risk factors or other potential confounders. The results of this study suggest that hyperglycemia-induced vascular oxidative stress may enhance MPO activity, thereby strengthening the negative impact of MPO on endothelium-dependent vasodilation.

In **chapter 6A** the association between MPO and blood pressure was investigated. MPO may influence blood pressure in at least two ways. First, MPO activity may scavenge the endogenous vasodilator nitric oxide. Second, MPO-derived reactive substances may damage the arterial wall, thereby reducing its elasticity. Indeed, our data demonstrated that a high plasma level of MPO was associated with a clinically relevant increase in both systolic and diastolic blood pressure. This association was strongest in subjects with (hyperglycemia-induced) oxidative stress, consistent with enhancement of MPO activity in the vasculature by increased local production of reactive oxygen species. These observations, together with emerging evidence that MPO-derived oxidants contribute to the initiation and propagation of CVD, identify MPO as a promising target for drug development. In **chapter 6B** we propose that, in addition to other mechanisms, direct inhibition of MPO and scavenging of MPO-derived reactive species are plausible mechanisms of how melatonin may protect the vasculature and contribute to lowering of blood pressure.

Production of nitric oxide by the endothelium is important for vascular function and is generally considered to counteract the process of atherogenesis. Arginine is the substrate for nitric oxide synthase (NOS), whereas asymmetric dimethylarginine (ADMA) is an endogenous competitive inhibitor of NOS. Therefore, a low arginine to ADMA ratio may be associated with reduced nitric oxide production, leading to endothelial dysfunction and accelerated atherogenesis. In **chapter 7** we investigated whether inflammation, which has been linked to both endothelial dysfunction and

increased CVD risk, is associated with altered concentrations of arginine and ADMA. For this purpose we evaluated two inflammatory markers, CRP and MPO, the latter being also directly related to oxidative stress. Increased levels of both MPO and CRP were associated with a lower arginine to ADMA ratio. In the case of MPO this was mainly due to an elevation of ADMA concentrations, wheras elevated CRP was associated with reduced arginine levels. With increasing plasma levels of oxLDL the positive association between MPO and ADMA was strengthened, compatible with amplification of oxidative stress by MPO. Notably, dimethylarginine dimethylaminohydrolase (DDAH), the enzyme responsible for degradation of ADMA, is highly sensitive to oxidative stress. Therefore, inhibition of DDAH by MPO-induced oxidative stress is a plausible mechanism by which MPO may cause increased ADMA levels. The negative relation between CRP and arginine was independent of oxLDL levels, suggesting involvement of inflammation rather than oxidative stress. A likely mechanism is increased consumption of arginine by the inducible form of arginase that is upregulated during inflammation. Taken together, the results described in this chapter support the notion that both MPO and CRP, by different mechanisms, are associated with reduced vascular nitric oxide production, possibly leading to endothelial dysfunction.

In **chapter 8** the relation between plasma levels of homoarginine and blood pressure was examined. Homoarginine has a strong structural resemblance to arginine. It has been shown that homoarginine can serve as substrate for NOS, but with less efficiency than arginine. In addition, homoarginine may compete with arginine for cell entry by the cationic amino acid transporters, leading to a reduced cellular uptake of arginine. Hence, we hypothesized that high homoarginine concentrations may result in reduced nitric oxide production by endothelial cells, possibly leading to increased blood pressure. In support of this hypothesis, we observed a positive, robust, and clinically relevant association between plasma levels of homoarginine and both systolic and diastolic blood pressure. In contrast, plasma levels of arginine were inversely associated with diastolic blood pressure, but only after adjusting for homoarginine concentrations. These observations lead to the conclusion that homoarginine and arginine may potentially act as antagonists in blood pressure regulation.

Conclusions

The first objective of this thesis was to study the relationships of novel markers of inflammation, oxidative stress, and nitric oxide signaling with vascular function and blood pressure.

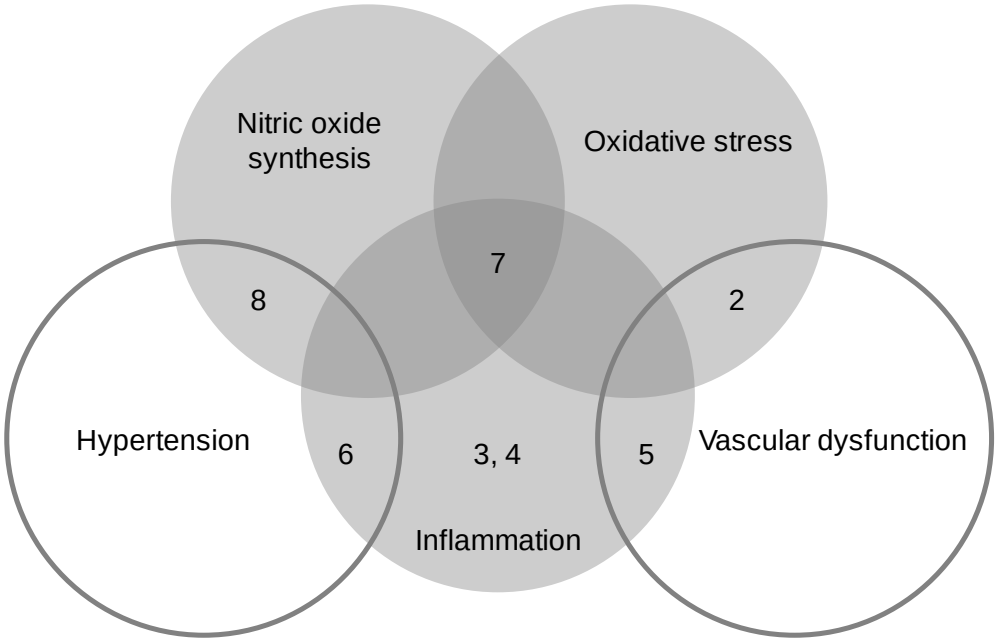

Figure Oxidative stress, inflammation, and a reduced nitric oxide synthesis (filled circles), alone and in conjunction, contribute to cardiovascular disease. We studied associations between markers of these processes and their associations with hypertension and vascular dysfunction (open circles). The numbers refer to the chapters in this thesis.

As graphically represented in the Figure, we observed associations between markers of inflammation (MPO and CRP) and key players of nitric oxide synthesis (the NOS substrate arginine and inhibitor ADMA). We also found both oxLDL and MPO to be associated with vascular dysfunction. Furthermore, MPO was associated with hypertension. Finally, a positive association between homoarginine and blood pressure was observed, that was antagonized by arginine.

Our second objective was to investigate whether these relationships are influenced by hyperglycemia and/or oxidative stress. This we could confirm, because oxidative stress and hyperglycemia strengthened the associations of MPO with blood pressure and vascular function. Also the positive association between MPO and ADMA was found to be enhanced by oxidative stress. Overall, the results of these studies confirm that relationships between biomarkers and measures of outcome

may be conditional, e.g. dependent on the level of oxidative stress or hyperglycemia. Acknowledgement of this fact may be crucial for interpreting differences between studies and study populations.

Study limitations and strengths

Our study population consisted of approximately equal-sized groups with normal glucose metabolism, impaired glucose metabolism, and type 2 diabetes mellitus. Therefore, the high prevalence of hyperglycemia may have caused a selection bias by favoring inclusion of individuals with a high cardiovascular risk. On the other hand this design made it possible to investigate hyperglycemia-related effect modification of the relation between biomarkers and measures of outcome. Since atherosclerosis is a disease that progresses with age, the observations within this elderly population may differ from those in younger subjects. Another limitation is that our epidemiological studies had a cross-sectional design and can therefore only reveal associations, which may or may not reflect causality.

The fact that the same cohort was used in our studies was very useful for reasons of comparability. Other strengths of the present study are the considerable number of participants included and the vast array of variables that were at our disposal to control for potential confounding.

Future perspectives

Plasma concentrations of oxLDL have been shown to be associated with CVD, but a comparison of the predictive power of oxLDL versus oxLDL/LDL-cholesterol or oxLDL/ApoB100 for CVD morbidity and mortality has never been performed. This would be interesting, since we observed that FMD was more strongly related with these ratios than with unadjusted oxLDL concentrations.

In several studies MPO has been found to be a significant predictor of CVD events. With increasing oxidative stress and glucose concentrations, we found stronger associations between MPO and blood pressure and vascular dysfunction. It would be worthwhile to investigate whether hyperglycemia or oxidative stress also strengthen the predictive power of MPO for (non)fatal CVD events. In addition, it may be interesting to compare the predictive values of serum, plasma, and leukocyte MPO concentrations as well as MPO activity for CVD events.

MPO contributes to hypertension and vascular dysfunction, but is also an important player in the innate immune system. Direct inhibition of MPO may therefore have positive cardiovascular effects, but on the downside lead to impairment of host defense. Reduction of oxidative stress, preferably locally in the vasculature, may be a better, more selective approach. Nitroxides, with Tempol as one of its best characterized members, form a promising class of compounds suitable to achieve this goal. Nitroxides are stable free radicals of low toxicity that exert a range of antioxidant effects, including catalytic dismutation of superoxide and breakdown of hydrogen peroxide. By reducing the levels of its cosubstrate hydrogen peroxide, MPO will be “starved” and no longer be able to produce hypochlorous acid and other reactive substances. In animal studies nitroxides have been shown to increase the bioavailability of nitric oxide and reduce blood pressure, and inhibition of the formation of hypochlorous acid by MPO has also been observed. Therefore, next to other beneficial effects, nitroxides hold potential to alleviate the adverse effects of MPO on blood pressure and vascular function.

We observed that homoarginine and arginine were antagonistically related to blood pressure, but the positive association between homoarginine and blood pressure was more pronounced than the negative relation between arginine and blood pressure. This is remarkable because circulatory concentrations of homoarginine are one to two orders of magnitude lower than concentrations of arginine. This novel finding warrants further investigation of the role of homoarginine in nitric oxide signaling and possibly as novel CVD risk marker or even risk factor.

Chapter 10

Nederlandse samenvatting en nabeschouwing

Het samenspel van oxidatieve stress en ontstekingsreacties in atherosclerose: een epidemiologische benadering

Samenvatting

Hoewel er veel vooruitgang is geboekt op het gebied van behandeling en preventie van hart- en vaatziekten (HVZ), is HVZ-gerelateerde sterfte nog steeds een van de belangrijkste doodsoorzaken in de westerse wereld. Ophoping van cholesterol in de vaatwand is een vroege stap in het ontstaan van atherosclerose. Ongemodificeerde (natieve) LDL deeltjes worden niet vastgehouden in de vaatwand. Alleen oxidatief beschadigde LDL deeltjes worden in overmaat opgenomen door macrofagen, die daardoor veranderen in schuimcellen. Door ophoping van deze schuimcellen ontstaat een “fatty streak”, die zich uiteindelijk kan ontwikkelen tot een atherosclerotische plaque. Acute cardiovasculaire gebeurtenissen, zoals een hartinfarct, ontstaan bij ruptuur van zo’n atherosclerotische plaque. Gedurende de afgelopen decennia is duidelijk geworden dat een verstoord vetmetabolisme (dyslipidemie) en verstoord glucose metabolisme (hyperglycemie) het proces van atherosclerose versnellen. Maar ook ontstekingsprocessen, oxidatieve stress en een verstoorde productie van stikstofoxide dragen bij aan atherosclerose, zowel bij verminderde endotheel-afhankelijke vaatfunctie, een vroeg proces in atherosclerose, als ook bij processen tijdens een gevorderd stadium van atherosclerose, zoals plaque ruptuur. Om inzicht te krijgen in de onderliggende processen in atherosclerose, hebben we diverse merkers voor oxidatieve stress, ontstekingsreacties en stikstofoxide vorming onderzocht in hun relatie met vaatfunctie en bloeddruk. Dit onderzoek is uitgevoerd in een cohort van ouderen, zowel met als zonder hyperglycemie (de Hoorn Studie).

In **hoofdstuk 2** hebben we onderzocht wat de determinanten zijn van geoxideerd LDL (oxLDL) in plasma en of er een associatie is tussen deze merker van oxidatieve stress en bloedstroom-gemedieerde vaatverwijdering (=flow-mediated dilation [FMD]) van de brachiale slagader. FMD is een merker voor endotheelfunctie en daarmee ook een vroege indicator van atherosclerose. De concentratie van oxLDL hangt niet alleen af van oxidatieve stress, maar ook van het aantal LDL deeltjes dat kan worden geoxideerd. Inderdaad werden sterke associaties tussen oxLDL en LDL-cholesterol en apolipoproteïne B-100 (ApoB100) gevonden. De sterke correlatie tussen deze variabelen maakt het moeilijk om hun onafhankelijke bijdrage aan het risico op HVZ te bepalen. Dit leidde ertoe dat wij de hypothese hebben onderzocht of de ratio’s oxLDL/LDL-cholesterol en oxLDL/ApoB100 informatiever zijn dan de afzonderlijke variabelen. Onze bevindingen onderschrijven deze hypothese,

omdat FMD van de brachialis niet was geassocieerd met oxLDL, LDL-cholesterol of ApoB100, maar wel significant negatief was geassocieerd met de ratio's oxLDL/LDL-cholesterol en oxLDL/ApoB100. De associaties tussen FMD en beide ratio's waren onafhankelijk van leeftijd, geslacht, Framingham risicoscore, nierfunctie en vetzucht. Hieruit zou kunnen worden geconcludeerd dat correctie van oxLDL voor het aantal LDL deeltjes de bruikbaarheid van de oxLDL meting in de klinische praktijk kan verbeteren.

In **hoofdstuk 3** wordt de bruikbaarheid van myeloperoxidase (MPO) in HVZ risicostratificatie besproken. Daartoe werden de resultaten van gepubliceerde cross-sectionele studies, case-control studies en prospectieve cohort studies geëvalueerd. De meeste van deze studies beschrijven een positieve associatie tussen hoge MPO concentraties en HVZ-gerelateerde morbiditeit en mortaliteit. De sterkste associaties werden waargenomen in populaties met acute cardiovasculaire symptomen. In meerdere studies werd aangetoond dat meting van MPO resulteerde in een betere risicostratificatie in vergelijking met traditionele HVZ merkers. Vervolgens worden in dit hoofdstuk kort de mechanismen waarlangs MPO kan bijdragen aan HVZ beschreven. Als onderdeel van het aspecifieke immuunsysteem vervult MPO een nuttige functie, namelijk het katalyseren van de vorming van zeer reactieve stoffen, zoals hypochloorzuur, die pathogene micro-organismen kunnen vernietigen. In het vaatsysteem daarentegen kunnen deze door MPO geproduceerde reactieve stoffen de vaatfunctie nadelig beïnvloeden en de structuur van de vaatwand aantasten. MPO reduceert de hoeveelheid stikstofoxide, is betrokken bij de oxidatie van LDL, tast HDL deeltjes aan en draagt door activatie van metalloproteïnases bij aan de verzwakking en ruptuur van plaques. Samenvattend kan worden gesteld dat MPO bijdraagt aan het ontstaan van HVZ en met name aan acute cardiovasculaire gebeurtenissen. Daarnaast wordt in dit hoofdstuk kort ingegaan op enkele technische aspecten van de MPO bepaling. In **hoofdstuk 4** wordt hier nader op ingegaan. In dit hoofdstuk worden serum, heparine-plasma en EDTA-plasma vergeleken als monstermateriaal voor de bepaling van MPO concentraties. In vergelijking met EDTA-plasma waren MPO concentraties in heparine-plasma twee keer zo hoog en in serum zelfs vier keer zo hoog. Bovendien werden er geen significante correlaties tussen de MPO concentraties in EDTA-plasma en heparine-plasma of serum gevonden. De verschillen in waargenomen MPO concentraties zijn mogelijk te verklaren door het ex vivo vrijkomen van MPO uit leukocyten, geïnduceerd door

heparine dan wel stolling. Dit ex vivo proces is moeilijk beheersbaar en daarom wordt EDTA-plasma aangeraden voor de bepaling van MPO concentraties in de circulatie.

In **hoofdstuk 5** is de associatie tussen MPO concentraties en de FMD van de brachiale slagader onderzocht. Met behulp van multivariate lineaire regressie analyse werden twee onafhankelijke determinanten van MPO gevonden: de dagelijkse inname van vitamine C en de plasma concentratie van C-reactief proteïne (CRP). Onze hypothese was dat het verband tussen MPO en FMD sterker zou zijn bij mensen met hyperglycemie, omdat hoog glucose samengaat met hogere concentraties van het essentiële MPO co-substraat waterstofperoxide. Inderdaad werd er een significante inverse associatie tussen MPO en FMD gevonden bij personen met een verstoord glucose metabolisme en dit was niet het geval bij mensen met een normaal glucose metabolisme. Deze associatie veranderde niet na correctie voor klassieke risicofactoren. Deze bevindingen suggereren dat hyperglycemie leidt tot verhoogde vasculaire oxidatieve stress wat de MPO activiteit versterkt en waardoor het negatieve effect van MPO op endotheel-afhankelijke vaatverwijding wordt verergerd.

In **hoofdstuk 6A** wordt de associatie tussen MPO en bloeddruk onderzocht. MPO zou op ten minste twee manieren de bloeddruk kunnen beïnvloeden. Ten eerste doordat bij MPO activiteit de endogene vaatverwijder stikstofoxide wordt verbruikt. Ten tweede kunnen door MPO geproduceerde reactieve stoffen de vaatwand beschadigen, wat leidt tot verminderde elasticiteit. Onze bevindingen tonen inderdaad aan dat hoge plasma MPO concentraties samengaan met klinisch relevante verhogingen van zowel systolische als diastolische bloeddruk. Deze associaties waren het sterkst in personen met oxidatieve stress, hetgeen in overeenstemming is met toegenomen MPO activiteit in de vaatwand door extra productie van reactieve zuurstofmoleculen. Deze waarneming, samen met het groeiende bewijs dat MPO producten bijdragen aan de initiatie en propagatie van HVZ, wijzen erop dat MPO een potentieel aangrijpingspunt zou kunnen zijn in de ontwikkeling van geneesmiddelen. In **hoofdstuk 6B** beargumenteren we dat, naast andere mechanismen, remming van MPO een plausibel mechanisme is voor de beschermende werking van melatonine op de vaatwand alsook voor het bloeddruk verlagende effect van melatonine. Behalve door directe remming van MPO kan melatonine deze effecten ook bewerkstelligen door het wegvangen van door MPO geproduceerde reactieve stoffen.

Productie van stikstofoxide door het endotheel is belangrijk voor een goede vaatfunctie en wordt beschouwd als een proces dat beschermt tegen atherogenese. Arginine is het substraat voor nitric oxide synthase (NOS) dat de vorming van stikstofoxide katalyseert, terwijl asymmetrisch dimethylarginine (ADMA) een endogene competitieve remmer is van NOS. Derhalve is een lage arginine/ADMA ratio mogelijk geassocieerd met verminderde stikstofoxide productie, wat kan leiden tot endotheeldisfunctie en versnelde atherogenese. In **hoofdstuk 7** onderzochten we of ontsteking, wat verband houdt met endotheeldisfunctie en een verhoogd risico op HVZ, is geassocieerd met afwijkende concentraties van arginine en/of ADMA. Hiertoe evalueerden we twee belangrijke ontstekingsmerkers, te weten CRP en MPO, waarvan de laatste ook een merker is voor oxidatieve stress. Zowel MPO als CRP waren negatief geassocieerd met de arginine/ADMA ratio. In het geval van MPO kwam dit door een positieve associatie tussen MPO en ADMA, terwijl dit in het geval van CRP werd veroorzaakt door een negatieve associatie met arginine. Bij een toename in oxidatieve stress werd de associatie tussen MPO en ADMA sterker, terwijl de associatie tussen CRP en arginine onveranderd bleef. Een mogelijke verklaring voor de positieve associatie tussen MPO en ADMA kan zijn dat DDAH, het enzym dat ADMA afbreekt, gevoelig is voor oxidatie en dat oxidatieve producten gemaakt door MPO, DDAH zouden kunnen inactiveren. De associatie tussen CRP en arginine lijkt eerder door ontstekingsreacties dan door aan oxidatieve stress gerelateerde processen te worden veroorzaakt. Extra consumptie van arginine door de induceerbare vorm van arginase, die wordt opgereguleerd tijdens ontstekingsprocessen, zou hiervoor een verklaring kunnen zijn. Samenvattend kunnen we concluderen dat zowel MPO als CRP, zij het via verschillende mechanismen, zijn geassocieerd met verminderde stikstofoxide productie, wat kan leiden tot endotheeldisfunctie.

In **hoofdstuk 8** wordt de relatie tussen de plasma concentratie van homoarginine en bloeddruk bestudeerd. Homoarginine lijkt qua structuur sterk op arginine en beide zijn substraat voor NOS, hoewel de effectiviteit van homoarginine als substraat veel lager is dan die van arginine. Tevens zijn homoarginine en arginine in competitie voor cellulaire opname via de kationische aminozuur transporters, waardoor een hoge homoarginine concentratie zou kunnen leiden tot een gereduceerde opname van arginine door de cel. Op deze wijze zou verhoogd homoarginine kunnen leiden tot verlaagde stikstofoxide productie door

endotheelcellen, resulterend in een verhoging van de bloeddruk. In overeenstemming met dit hypothetische mechanisme vonden wij een robuuste- en klinisch relevante positieve associatie van homoarginine met zowel diastolische als systolische bloeddruk. Arginine was daarentegen negatief met diastolische bloeddruk geassocieerd, maar alleen na correctie voor homoarginine concentraties. Uit deze studie blijkt dat homoarginine en arginine mogelijk als een antagonistisch koppel betrokken zijn bij bloeddrukregulatie.

Conclusies

Dit promotieonderzoek had twee doelstellingen. De eerste doelstelling was het onderzoeken van de relaties van nieuwe merkers voor ontstekingsreacties, oxidatieve stress en stikstofoxide productie met vaatfunctie en bloeddruk. Zoals weergegeven in het figuur op pagina 178 vonden we associaties tussen merkers van inflammatie (MPO en CRP) en belangrijke componenten van stikstofoxide synthese (NOS substraat arginine en NOS remmer ADMA). Tevens vonden we dat vaatfunctie invers was geassocieerd met oxLDL en MPO. Daarnaast was er een positief verband tussen MPO en bloeddruk, en bleken arginine en homoarginine mogelijk antagonisten te zijn in de bloeddrukregulatie. De tweede doelstelling was het onderzoeken of de gevonden associaties werden beïnvloed door oxidatieve stress en/of hyperglycemie. Inderdaad werden de associaties tussen MPO en bloeddruk en vaatfunctie versterkt door zowel oxidatieve stress als hyperglycemie. Ook de associatie tussen ADMA en MPO bleek sterker bij oxidatieve stress. Alles overwegend laten de resultaten van dit proefschrift zien dat de relaties tussen de bestudeerde biomerkers en maten van uitkomst conditioneel kunnen zijn, namelijk afhankelijk van oxidatieve stress en het glucose metabolisme. Onderkenning van dit gegeven kan van belang zijn voor de juiste interpretatie van onderzoeksuitkomsten.

Beperkingen en sterke punten van de studies

De onderzochte populatie bestond uit ongeveer even grote groepen met een normaal glucose metabolisme, een verstoord glucose metabolisme (“impaired glucose metabolism”) en met type 2 diabetes. Door de hoge prevalentie van hyperglycemie in onze studiegroep zijn relatief veel personen met een hoog HVZ risico geïncludeerd, waardoor onze resultaten mogelijk niet representatief zijn voor de algemene

bevolking. Anderzijds maakte het grote aantal mensen met hyperglycemie het mogelijk om het effect van hyperglycemie op de verbanden tussen biomerkers en uitkomstmaten te bestuderen. Atherosclerose is een ziekte die met de leeftijd voortschrijdt en daarom is het mogelijk dat onze bevindingen in deze wat oudere populatie anders zijn dan in jongere mensen. Daarnaast is het zo dat de Hoorn Studie een epidemiologische studie is die een dwarsdoorsnede van de populatie beschrijft en geschikt is om associaties te vinden, maar om te kunnen bevestigen of deze associaties een causaal verband weerspiegelen is aanvullend onderzoek nodig.

Het feit dat steeds hetzelfde cohort is gebruikt, maakt vergelijking tussen onze studies goed mogelijk. Andere sterke punten zijn het aanzienlijke aantal deelnemers en de vele klinische en biochemische variabelen die ons ter beschikking stonden om als covariabelen in statistische modellen te gebruiken.

Vooruitblik

Hoewel er positieve associaties tussen oxLDL concentraties in plasma en HVZ zijn beschreven, is er nog geen vergelijkend onderzoek gedaan naar de voorspellende waarde van oxLDL versus oxLDL/ApoB en oxLDL/LDL-cholesterol voor HVZ-gerelateerde morbiditeit en mortaliteit. Dit zou interessant kunnen zijn, omdat in ons onderzoek deze verhoudingen sterker geassocieerd waren met vaatfunctie dan oxLDL zelf.

MPO is in diverse studies een voorspeller van HVZ gebleken. Wij vonden dat met toenemende oxidatieve stress en bloed glucose concentraties het verband tussen MPO en bloeddruk en vaatdisfunctie sterker werd. Daarom is het interessant om te onderzoeken of hyperglycemie en oxidatieve stress de voorspellende waarde van MPO voor (fatale) cardiovasculaire gebeurtenissen verhogen. Bovendien is het interessant om de voorspellende waarde van MPO concentraties bepaald in serum, plasma en leukocyten en ook MPO activiteit voor cardiovasculaire gebeurtenissen te vergelijken.

MPO draagt bij aan hoge bloeddruk en vaatdisfunctie, maar is ook van belang voor de aspecifieke afweer. Directe remming van MPO heeft daarom mogelijke positieve effecten op hart en vaten, maar leidt daarnaast wellicht ook tot een verminderde afweer. Reductie van oxidatieve stress, bij voorkeur lokaal in de vaatwand, is mogelijk een meer selectieve en betere aanpak. Nitroxides, waarvan

Tempol een van de best gekarakteriseerde is, zijn een groep geneesmiddelen die geschikt lijkt voor dit doel. Nitroxides zijn stabiele vrije radicalen met een lage toxiciteit die op een aantal manieren als antioxidanten werken, onder meer door katalytische dismutatie van superoxide en afbraak van waterstofperoxide. Door het wegvangen van waterstofperoxide, het essentiële co-substraat van MPO, kan MPO niet langer de synthese van zeer reactieve producten zoals hypochloorzuur katalyseren. In studies in dieren is waargenomen dat nitroxides de beschikbaarheid van stikstofoxide verhogen, de bloeddruk verlagen en de vorming van hypochloorzuur door MPO remmen. Daarom hebben nitroxides mogelijk een beschermende werking tegen door MPO gemedieerde bloeddrukverhoging en vaatdisfunctie.

Uit onze studies blijkt dat homoarginine en arginine op antagonistische wijze gerelateerd zijn met bloeddruk. De positieve associatie van homoarginine met bloeddruk was meer uitgesproken dan de negatieve relatie tussen arginine en bloeddruk. Dit is opmerkelijk, omdat de concentratie van homoarginine in de circulatie een factor tien tot honderd lager is dan die van arginine. Deze observatie is nieuw en vraagt om meer onderzoek naar de rol van homoarginine in stikstofoxide signalering en als mogelijk nieuwe HVZ risicomerker of zelfs risicofactor.

Dankwoord

Epidemiologische studies zoals in dit proefschrift beschreven kunnen alleen worden gedaan omdat mensen bereid zijn deel te nemen. Daarvoor wil ik dan ook de deelnemers van het Relat-2 cohort (Hoorn Study en Hoorn Screening Study) bedanken.

In hoofdstuk 4 hebben we de geschiktheid van EDTA-plasma, heparine plasma en serum vergeleken voor de bepaling van MPO. Daarvoor hebben diverse mensen hun bloed afgestaan. Dank daarvoor.

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Leonard Peter van der Zwan was born on December 4th 1975 in Ten Boer, The Netherlands. As secondary education a HAVO degree was obtained in 1994 and subsequently a VWO degree in 1996. For a year he studied economics at the Erasmus University in Rotterdam, before switching to study chemistry at Utrecht University, Utrecht in 1997. An external internship was performed at Sanquin Research (formerly known as CLB) in Amsterdam. He completed his chemistry study in 2004. From 2005 to present he was a Ph.D. student at the Metabolic Laboratory of the Clinical Chemistry Department of the VU University Medical Center in Amsterdam.